GENETIC MOUSE MODELS ELUCIDATE THE ROLES OF ADRENOMEDULLIN IN CARDIOVASCULAR DEVELOPMENT AND PHYSIOLOGY

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ABSTRACT

William Paul Dunworth: Genetic Mouse Models Elucidate the Role of Adrenomedullin in Cardiovascular Development and Disease

(Under the direction of Dr. Kathleen M. Caron)

Adrenomedullin (AM) is a highly conserved, secreted 52 amino acid peptide that functions in physiological processes within the nervous, reproductive, and cardiovascular systems. AM is nearly ubiquitously expressed, but most highly expressed from the vasculature, lungs, and heart. During cardiovascular stresses including myocardial infarction, hypertension, renal failure and normal pregnancy, AM serum levels are elevated suggesting that AM functions as a cardioprotective factor. AM signals through a unique paradigm of G-protein coupled receptor (GPCR) signaling in which the affinity of calcitonin receptor-like receptor (CLR=protein, *Calcrl*=gene) for its ligands, AM or calcitonin gene-related peptide, is dictated by a family of single-pass transmembrane proteins called receptor activity modifying proteins (RAMPs). Ramp2-CLR and Ramp3-CLR specifically bind AM while RAMP1-CLR functions as the CGRP receptor. Research presented in this dissertation aims to further our understanding of AM signaling in mammalian cardiovascular physiology through the utilization of genetic mouse models and in vitro approaches.

I demonstrate that gene-targeted knockout mice of *AM*, *Calcrl*, and *Ramp2* are embryonic lethal from specific defects in lymphatic endothelial cell (LEC) proliferation resulting in generalized interstitial edema. Within this study, I present a model in which AM signaling components are enriched during LEC differentiation which, in the absence of AM signaling, explains the observed hypoplastic lymphatic vascular development. I also investigated whether a loss in LEC barrier function could have contributed to this phenotype. While I found no difference in the ultra structural features or expression of LEC junctional components in knockout mice, I show that in vivo and in vitro AM stabilizes the LEC barrier and can completely abrogate the highly permeabilizing actions of vascular endothelial growth factor A, supporting an important role of AM signaling in LEC barrier regulation. In a separate study, I generated and characterized a vascular smooth muscle cell-specific *Calcrl* deficient mouse. These mice survived to adulthood with no defects in the regulation of basal blood pressure or cardiovascular function which may be due to compensation by other hemodynamic mechanisms. Together, these studies have built a solid foundation that will one day benefit future clinical applications of AM in lymphatic and vascular smooth muscle pathologies.

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TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

LIST OF ABBREVIATIONS

AC	adenylate cyclase
Akt	protein kinase B
AM	adrenomedullin
BNP	brain natriuretic peptide
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
Ca^{+2}	calcium ion
calcrl	calcitonin receptor-like receptor gene
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene related peptide
CLR	calcitonin receptor-like receptor protein
CTR	calcitonin receptor
E. coli	*Escherichia coli*
EC	endothelial cells
EGFP	enhanced green fluorescent protein
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinases
ES cells	embryonic stem cells
F-actin	filamentous actin
FAK	focal adhesion kinase
FBS	fetal bovine serum

FOXC2	forkhead box C2
GC	guanylate cyclase
GPCR	G-protein coupled receptor
H&E	hematoxylin and eosin
hAM	human adrenomedullin
hCALCRL	human *calcitonin receptor-like receptor* gene
hRAMP	human *receptor activity modifying protein* gene
HMVEC-dLys	human dermal lymphatic microvascular endothelial cells
HUVECs	human umbilical vein endothelial cells
IL-1β	interleukin-1 beta
JAMC	junction adhesion molecular C
K^+	potassium ion
LECs	lymphatic endothelial cells
L-NAME	N-nitro-L-arginine methyl ester
LYVE1	lymphatic-endothelial-hyaluronan-receptor-1
MAPK	mitogen-activated protein kinase
NO	nitric oxide
PAM	peptidyl glycine α monooxygenase
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween 20
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDZ	Post synaptic density protein, Drosophila disc large tumor suppressor (DlgA), and Zonula occludens-1 protein (zo-1)

PECAM	platelet/endothelial cell adhesion molecule-1
pERK	phosphorylated extracellular signal-regulated kinases
PKA	protein kinase A
PFA	paraformaldehyde
Prox1	prospero-related homeobox 1
PTH	parathyroid hormone
RAMP1, 2 and 3	receptor activity modifying protein 1, 2 and 3
RT-PCR	reverse transcription-polymerase chain reaction
S. aureus	*Staphylococcus aureus*
SLP76	SH2-domain-containing leukocyte protein of 76 kDa
SM22	smooth muscle 22α
Sox18	SRY (sex determining region Y)-box 18
Spred1	sprouty protein-related with EVH1 domain 1
Spred2	sprouty protein-related with EVH1 domain 2
Syk	Spleen tyrosine kinase
TBST	tris buffered saline with Tween 20
Tie2	angiopoietin receptor 2
TNF-α	tumor necrosis factor-alpha
VIP/VPAC1	vasointestinal peptide/pituitary adenylate cyclase activating peptide
VEGFA	vascular endothelial growth factor A
VEGFC	vascular endothelial growth factor C
VEGFD	vascular endothelial growth factor D
VEGFR3	vascular endothelial growth factor receptor 3

VPAC1	vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor
VSMC	vascular smooth muscle cells
WT	wild-type
ZO-1	zonula occludens 1

CHAPTER 1

INTRODUCTION

The Multifunctional Adrenomedullin Peptide

Adrenomedullin (AM) is a highly conserved 52 amino acid peptide and a member of the calcitonin peptide superfamily which also includes calcitonin gene-related peptide (CGRP), amylin, intermedin, and calcitonin. The AM secondary structure includes a 6 amino acid ring formed by disulfide bonds between residues 16 and 21. Also, the C-terminal tyrosine residue is amidated by peptidyl glycine α monooxygenase (PAM) [1]. Both of these features are necessary to confer the biological activity of AM.

Adrenomedullin was initially isolated from human pheochromocytoma and noted for its ability to increase cAMP levels in human platelets and cause a potent hypotensive response in rats [2]. AM is expressed and secreted by nearly all mammalian tissues and cell types but is most strongly expressed in the endothelial cells and vascular smooth muscle cells of the heart [3], lungs [4], and vasculature [5, 6]. As a circulating hormone, plasma levels of AM have been found in the concentration range of 2 -10 picomolar [7]. In addition to its aforementioned function as a vasodilator, AM is involved in angiogenesis [8], vascular permeability [9], growth and apoptosis [10], renal function [11], bronchodilation [12] , neurotransmission [13], and anti-microbrial defense [14]. Several factors stimulate AM production and secretion including hypoxia [15], oxidative stress [16], sheer stress [17], vasoconstrictors such as endothelin-1 and angiotensin II [18], and inflammatory cytokines such as TNF-α, IL-1β, and interferon-1γ [19].

As shown in **Figure 1.1**, which summarizes findings from over 60 independent investigations, plasma levels of AM are significantly increased in a wide

range of physiological stresses from normal pregnancy to cancer. Of great significance, plasma levels of AM are elevated in many forms of cardiovascular disease including arterial hypertension [20], sepsis [21], congestive heart failure [3], myocardial infarction [22] and renal disease [23] which together suggest a protective role for AM in the cardiovascular system. However, exactly how AM elicits its cardioprotective effects remains largely unknown and subsequently, represents an active field of research in hopes of manipulating AM function in a clinical setting.

AM Signaling Through a Unique GPCR Paradigm

The mechanism through which AM elicits its cellular effects represents a unique system of G protein-coupled receptor (GPCR) signaling. Initially, three putative orphan receptors (calcitonin receptor-like receptor (CLR=protein; *Calcrl*=gene name), L1, and RDC-1) were receptor candidates for AM since they could bind AM at similar affinities and could generate a dose-dependent cAMP response [24-28]. However, a consensus as to which receptor was the putative AM receptor could not be reached due to conflicting data and irreproducible results [27, 29-31]. At last, CLR was recognized as the most likely AM receptor after the discovery of a novel class of single-transmembrane domain proteins called receptor activity modifying proteins (RAMPs), which helped to demystify the confusion surrounding the AM receptor [31]. From this landmark discovery, it was determined that RAMPs dictate ligand specificity by associating with CLR at the plasma membrane. As depicted in **Figure 1.2**, a RAMP1-CLR heterodimer confers a receptor for calcitonin gene-related peptide (CGRP) while a RAMP2-CLR or

RAMP3-CLR heterodimer functions as a receptor for AM. Therefore, the spatial and temporal expression patterns of the RAMPs determine the cellular response to either AM or CGRP. It should be noted that since the discovery RAMPs, several other class II GPCRs have been demonstrated to interact with RAMPs in a similar fashion including calcitonin receptor, glucagon receptor, and vasoactive intestinal peptide/pituitary adenylate cyclase activating peptide 1 receptor, among others [32, 33].

Our laboratory was the first to use gene-targeted knockout mouse models to confirm the identity of the functional AM receptor. Specifically, deletion of *AM*, *Calcrl*, or *Ramp*2 resulted in embryonic lethality at mid-gestation from generalized edema and severe cardiovascular defects including hypoplastic lymph sacs, thinner vascular smooth muscle walls, and disorganized, smaller hearts [34-36]. The fact that all 3 KO mouse models had similar phenotypes provided the first in vivo genetic evidence that RAMP2-CLR functions as a bonafide AM receptor during development. Further, the similarity of the AM signaling null mouse phenotype with other mouse models describing defects in lymphatic vascular development, such as the Prox1 and VEGFC null mice suggested to us that AM signaling may play an essential role in this process [37, 38].

AM in Endothelial Cell Biology

The vascular endothelium consists of a thin layer of endothelial cells (ECs) that line the interior of the entire circulatory system, from the large coronary vessels of the heart to the smallest capillaries. The main function of the circulatory system is

to maintain homeostasis by coordinating proper gas, nutrient, and waste exchange to and from cells. ECs are a specialized type of epithelium that differentiate from endothelial progenitors during early embryonic development, in a process termed vasculogenesis, to form the primary vascular plexus. Interestingly, AM has been shown to promote arterial differentiation of cultured mouse embryonic stem cells through a cell signaling cascade that involves cAMP-mediated activation of Notch signaling [39]. Furthermore, down regulation of CLR in zebrafish resulted in loss of arterial identity of the dorsal aorta, suggesting that AM signaling through CLR plays a non-redundant role in arterial differentiation [40].

Vasculogenesis is followed by angiogenesis, which by definition includes the sprouting and branching of pre-existing vessels to form the functional vascular network. At the cellular level, angiogenesis includes the processes of EC proliferation, migration, and tube formation and remodeling. AM acts on ECs in an autocrine / paracrine manner as an angiogenic factor [41]. Using cultured human umbilical vein endothelial cells (HUVECs), Miyashita *et al* showed that AM was a strong proliferative and migratory factor [42]. AM can also induce HUVEC differentiation to tube-like structures on Matrigel [8]. These cellular effects occur through several downstream intracellular pathways, including activation of mitogen-activated protein kinase (MAPK), Akt, and focal adhesion kinase (FAK) [43, 44]. These in vitro results have been recapitulated in vivo by Iimuro *et al* where AM treatment increased capillary density in both the hind limb ischemia model and transplanted tumors [45]. Interestingly, AM administration increased the local expression of vascular endothelial growth factor (VEGF), a finding supported by in

vitro studies [45, 46]. This finding, together with the observation that AM heterozygote (AM+/-) mice express lower amounts of VEGF [45], strongly suggest that a synergistic relationship between exists AM and VEGF. VEGF is known as the most potent and predominant angiogenic factor. Recently, it has been suggested that AM signaling through RAMP2-CLR could transactivate the VEGF receptor leading to increased downstream angiogenic effects [47]. Exactly how AM and VEGF interact molecularly remains an intense area of investigation since both pro-angiogenic factors are highly expressed in many forms of cancer and have prognostic value (rev. [48, 49]).

AM also functions as an anti-apoptotic, pro-survival factor in ECs. Notably, both *AM* and *Calcrl* contain hypoxia-response elements in their promoter regions, which increases their gene expression during low oxygen conditions, thereby promoting EC survival, as well as angiogenesis [50, 51]. Several studies have elucidated the mechanisms through which AM functions to prevent EC death. A study by Kato *et al* demonstrated that AM could promote cell survival of rat ECs in a mechanism independent of the activation of adenylate cyclase and cAMP [52]. Utilizing HUVECs, Sata *et al* found that the anti-apoptotic function of AM was cGMP independent but could be repressed by nitric oxide (NO) inhibitor L-NAME, suggesting a NO-dependent mechanism [53]. More recently, it was found that AM treatment of serum-deprived ECs increased the expression of Max, a transcription factor that heterodimerizes with c-Myc to promote cell survival [54]. Therefore, it appears that the intracellular signaling mechanisms that regulate AM-mediated EC

proliferation and survival may differ, which highlights the multi-functional role and complexity of AM in EC biology.

AM has potent effects on the establishment and maintenance of the EC barrier and is therefore a strong regulator of endothelial permeability. Loss of EC barrier function is a key characteristic of acute inflammation that can proceed to organ dysfunction and severe infection. AM has been shown to inhibit vascular leakage in response to the dramatic dysequilibrium encountered in cardiovascular diseases such as sepsis and inflammation [9]. Specifically, AM reduced endothelial hyperpermeability of HUVECs stimulated with permeability increasing agents including hydrogren peroxide, thrombin, *E. coli* hemolysin, and *S. aureus* α–toxins [9]. Further, AM has been found to effectively tighten the blood brain barrier in experiments utilizing cerebral ECs [55]. This endothelial stabilizing function of AM has been shown in vivo as well. In rats subjected to septic shock by intravenous *S. aureus* α-toxin administration, pre-treatment with AM was able to prevent associated hyperpermeability of the lung, liver, ileum, and kidney [56]. Mechanistically, it has been suggested that AM elicits these cellular effects through increasing intracellular cAMP, which in turn stabilizes junctional complexes between ECs as well as reduces cell contraction by preventing the formation of F-actin stress fibers by inhibiting myosin-light chain phosphorylation (rev. [57]).

Taken together, these data suggest that AM signaling in the blood vasculature contributes to its proper development, remodeling, and function. Work presented in this thesis demonstrates that AM signaling also plays an important role in the development and function of the lymphatic vascular system. Mouse embryos

lacking AM or its receptors develop hypoplastic lymph sacs as a result of reduced proliferation of lymphatic endothelial cells (LECs) [36]. In another study contained within this thesis, AM was shown to inhibit VEGF-mediated increases in LEC permeability in vitro and in vivo [58].

AM as a Cardioprotective Factor in Vascular Smooth Muscle Cells

AM functions as a cardioprotective factor during hypertension. Indeed, plasma levels of AM are increased in hypertensive patients and correlate with disease severity (**Figure 1.1**) [20, 59, 60]. Since hypertension can lead to impaired kidney function, a correlation also exists between AM plasma levels and impaired renal function and renal failure [60]. In patients with complications from primary arterial hypertension, including left ventricular hypertrophy, AM plasma levels are elevated as well [20]. More recently, a clinical trial showed that plasma AM has better prognostic value than brain natriuretic peptide (BNP) following myocardial infarction, therefore the clinical interest in AM during cardiovascular disease is high [61].

Perhaps the most recognized function of AM is to act as a potent vasodilator; with many studies finding that AM causes both a dose-dependent and prolonged hypotensive response in variety of tissues and species [62-67]. Up-regulation of AM during hypertension can be cardioprotective by eliciting vasodilation and natriuresis, thereby reducing myocardial hypertrophy and remodeling. For example, adenoviral-mediated delivery of AM significantly reduced blood pressure and limited cardiac and renal structural and functional damage in several rat models

of hypertension including the Dahl salt-sensitive model, and deoxycorticosterone acetate-salt model [68-70].

At the molecular level, AM signaling can elicit vasodilation and relaxation of the vascular smooth muscle wall two ways: i) directly through CLR activation in VSMCs leading to stimulation of protein kinase A (PKA) which in turn causes smooth muscle relaxation by opening K^+ channels and activation of Ca^{+2} sequestration mechanisms or ii) indirectly through binding to CLR in ECs to initiate the secretion of NO which acts upon VSMC to cause relaxation (**Figure 1.3**) [71-73]. However, because the studies leading to these mechanisms utilized different species, vessels, and methods, the precise mechanisms responsible for the hypotensive effects of AM have yet to be determined [74-76].

To address these issues, the Caron laboratory has generated genetic mouse models with either haploinsufficiency or tissue-specific deletion of AM and its receptors. These studies have consistently shown that while genetic reduction of AM or its receptor does not affect basal or stress-induced vascular tone in adult mice, loss of AM signaling causes reduced VSMC proliferation during embryonic development [34, 35, 77, 78]. The finding that AM promotes VSMC growth and proliferation is supported by numerous other studies [79-81]. However, the lack of a robust phenotype on VSMC function may be due to the modest effects resulting from a haploinsufficient model. Work presented in this thesis shows that genetic deletion of CLR in VSMCs is compatible with survival and does not affect basal vascular tone, suggesting either compensation by other vasodilators or that signaling of AM and/or CGRP through CLR in ECs is sufficient to mediate vasodilation.

Research Presented in this Dissertation

As described in the preceding sections, AM certainly influences many aspects of cardiovascular physiology. Previous studies performed in the Caron laboratory sought to investigate the effects of loss of AM signaling during development by generating gene-targeted knockout mouse models of *AM*, *Ramp2*, and *Calcrl*. These studies showed that AM signaling is essential for survival, since each of the genetic null mice were embryonic lethal at mid-gestation with severe defects in cardiovascular development [34, 35, 82]. The overall goals of this dissertation were to further characterize these cardiovascular defects with specific focus on the role of AM in both the development and function of the lymphatic vascular system and VSMCs. Chapter 3 describes the discovery of a defect in LEC proliferation in mice globally lacking AM signaling (*AM*, *Ramp2*, *Calcrl* null mice) and specifically in ECs (EC-specific deletion of *Calcrl* mice). Further, complimentary in vitro studies provided a mechanism for which enhanced AM signaling during the proliferation steps of lymphangiogenesis is necessary for proper lymphatic vascular development. Chapter 4 describes an investigation into the role of AM in regulating lymphatic permeability in vivo and in vitro. AM stimulation was shown to strengthen the LEC barrier during permeabilizing conditions by reorganizing and stabilizing junctional complexes. Chapter 5 describes the generation and characterization of mice lacking *Calcrl* expression specifically in VSMCs, while maintaining normal expression levels in all other cell types. These experimental mice were born at expected Mendalian ratios and survived to adulthood with no obvious cardiovascular defects when investigated for blood pressure, heart function, or metabolic function.

Collectively, this dissertation explores the role of AM signaling during development and adulthood in a cell- specific manner.

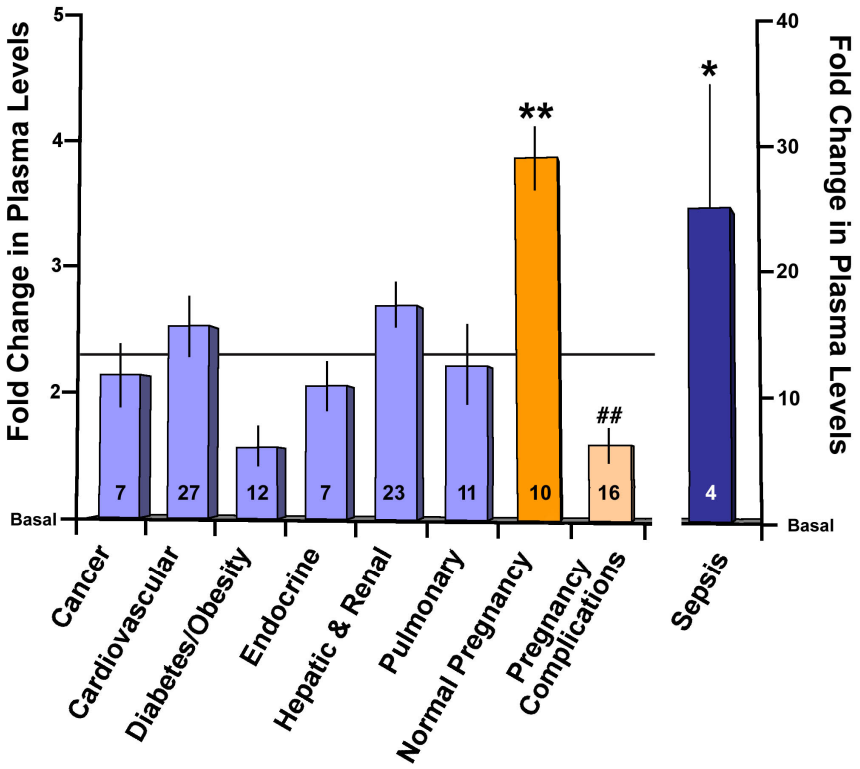

Figure 1.1: Fold change in plasma AM levels in a variety of human conditions. *Bars* indicate average fold change in circulating AM levels in various disease categories or conditions based on published human clinical data. The *horizontal line* at 2.3 represents the average fold increase in plasma AM levels across all conditions (excluding sepsis). ******, $P < 0.007$ between pregnancy and all other disease conditions (excluding sepsis). ##, $P < 0.001$ between normal pregnancy and pregnancy complications. *****, $P < 0.05$ between sepsis and all other conditions. *Number on each bar* indicates the number of published observations assessing plasma AM levels in each category.

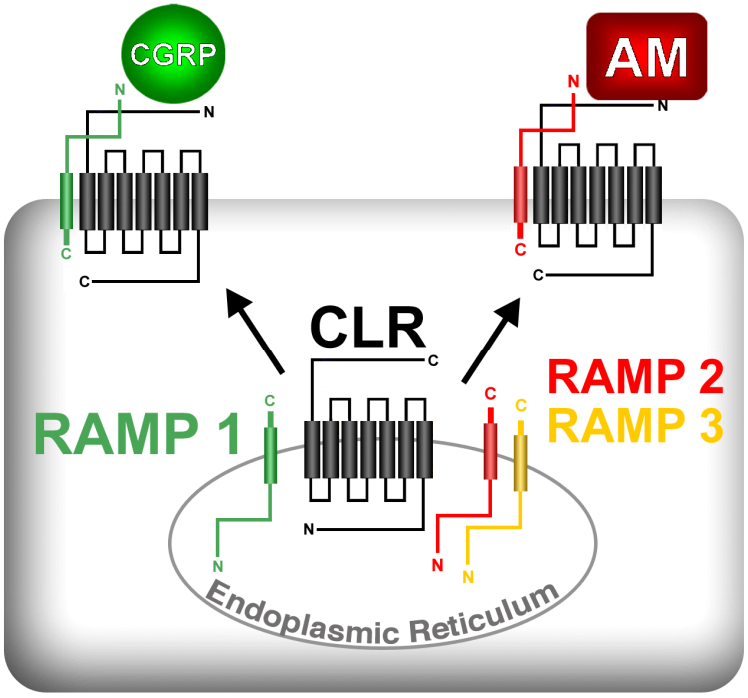

Figure 1.2: The RAMP/Receptor paradigm for AM and CGRP signaling. RAMPs convey receptor specificity by heterodimer formation with CLR in the endoplasmic reticulum followed by localization to the plasma membrane. The association of CLR with a RAMP determines the specificity of ligand binding. Thus, a RAMP1-CLR heterodimer (*green*) binds preferentially to CGRP, whereas association of CLR with either RAMP2 (*dark red*) or RAMP3 (*light yellow*) results in preferential binding to AM.

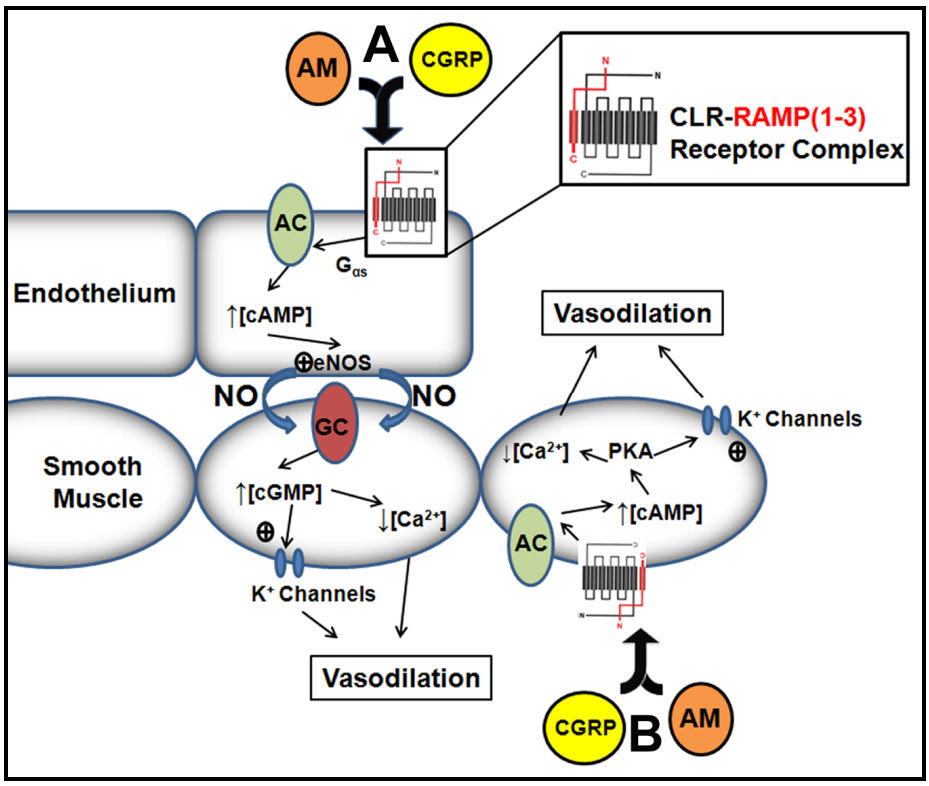

Figure 1.3: Cellular mechanisms of vasodilation induced by AM or CGRP. AM binds to RAMP2/3-CLR and CGRP binds to RAMP1-CLR in ECs and VSMCs leading to relaxation of the VSMC wall. In the EC-dependent pathway ,(**A**), ligand binding activates adenylate cyclase (AC) leading to the increased production of the second messenger cAMP which, in turn, stimulates NO synthesis and release to the adjacent VSMC. NO activates guanylate cyclase (GC) that directs cGMP production which subsequently relaxes the VSMC by activating Ca^{2+} sequestration mechanism and opening K^+ channels. In the EC-independent pathway, (**B**), activation of receptor on VSMCs by ligands is coupled to cAMP production by adenylate cyclase. cAMP activates PKA which opens K^+ channels and decreases intracellular Ca^{2+}calcium levels, leading to VSMC relaxation. Adapted from [83].

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CHAPTER 2

THE LYMPHATIC VASCULAR SYSTEM IN DEVELOPMENT AND DISEASE: THE POTENTIAL FOR ADRENOMEDULLIN-BASED THERAPIES

The Lymphatic Vascular System & Lessons from Genetic Mouse Models

The lymphatic vascular system works to maintain tissue fluid homeostasis, absorb fatty acids and lipid-soluble vitamins from the gut and traffic antigen-presenting immune cells through the lymph nodes. The lymphatic vasculature is a blind-ended network of endothelial cell lined vessels that unidirectionally transports tissue fluid, extravated plasma proteins, lipids and cells from the interstitial space back to the circulatory system via the thoracic duct into the subclavian vein. The lymphatic vascular system can be divided into two distinct segments, the initial lymphatics and the collecting lymphatics. The initial lymphatics are relatively small vessels, consisting of a single layer of endothelial cells. They lack both a basement membrane and smooth muscle cell coverage. The initial lymphatics feed into the collecting lymphatics, which are lined by a layer of lymphatic vascular smooth muscle cells and pericytes. The collecting lymphatics generate lymph flow through both tonic and phasic contractions (rev. in [1]). Recently, understanding how the lymphatic vascular system develops and functions has been an area of intense research as investigators looks to discover new avenues for treating lymphatic diseases.

Florence Sabin is credited with proposing the current model of lymphatic vascular development over 100 years ago [2]. Through experimentation with fetal pigs, Sabin's findings suggested that predetermined endothelial cells bud off from pre-existing veins to form primary lymph sacs, which in turn proliferate and branch throughout the periphery to form lymphatic vessels and capillaries. Only recently has Sabin's model gained wide acceptance due to advances in genetic engineering

techniques that have identified series of genes that regulate lymphatic vascular development in a stepwise manner (rev. in [3]). The impact that gene targeting has had on driving the field forward is unparalleled, and so it is fitting that the 2007 Nobel Prize in Physiology or Medicine was awarded to Smithies, Capecchi and Evans in recognition of their efforts toward developing gene targeting approaches.

Figure 2.1 summarizes our current understanding of the stepwise process of lymphangiogenesis and lists numerous genetic factors that have been identified as important for lymphangiogenesis through gene targeting approaches. Development of the lymphatic vascular system is initiated when lymphatic-endothelial-hyaluronan-receptor-1 (LYVE1)-expressing endothelial cells of the cardinal vein begin to express the transcription factor sex determining region Y-box 18 (Sox18) which drives expression of prospero-related homeobox 1 (Prox1) in a polarized manner [4]. Sox18 and Prox1 transcriptionally reprogram venous endothelial cells so that they become specified toward a lymphatic fate. As shown in **Figure 2.2A-B**, mice lacking either *Sox18* or *Prox1* are embryonic lethal at midgestation, develop with a complete absence of differentiated lymphatic endothelial cells (LECs), and display generalized interstitial edema [4, 5].

After lymphatic endothelial competence has been established, the up-regulation of the vascular endothelial growth factor C (VEGFC)-receptor, VEGFR3, in LECs confers their ability to sprout from the cardinal vein and migrate toward an ectopic gradient of growth-promoting VEGFC. *Vegfc* null mice fail to develop a lymphatic vascular system and die from severe edema at e15.5 [6] (**Figure 2.2C**). More recently, mice with double genetic deletion of sprouty protein-related with

EVH1 domain 1 and 2 (*Spred1/ 2*) (negative regulators of growth factor-induced MAPK/ERK activation) develop marked edema, dilated lymphatic vessels and a hyperproliferation of LECs due to abnormal loss of VEGFC/VEGFR-3 inhibition [7] (**Figure 2.2D**). These studies, together with work presented in this thesis, support an essential role for the MAPK/ERK pathway in lymphangiogenesis.

Subsequent rounds of proliferation lead to the formation of a primary lymphatic plexus which is later remodeled with mural cells and luminal valves to form the fully functional lymphatic vascular network. Little is known about the processes that regulate lymphatic vessel maturation and patterning during the later stages of embryonic development or during the early neonatal period. Mice lacking the glycoprotein podoplanin die neonatally from lymphedema as a result of severe lymphatic vessel disorganization, dilated vessels, and diminished lymphatic transport [8] (**Figure 2.2E**). Mice, as well as humans, deficient for the transcription factor forkhead box C2 (FoxC2) have abnormal lymphatic vascular patterning, lack lymphatic valves, and acquire aberrant smooth muscle coverage on the initial lymphatics [9]. Further, mice lacking a functional PDZ domain of the ephrinB2 protein display irregular postnatal remodeling of the lymphatic vasculature [10]. While these data are a step forward in our understanding of this process, they do not describe molecular interactions responsible for proper lymphatic patterning and postnatal maturation. This thesis dissertation describes an investigation into the role of adrenomedullin signaling through its receptor CLR in lymphatic vascular development, specifically during the processes of LEC proliferation after the formation of the primary lymph sac.

Diseases of the Lymphatic Vascular System

Diseases of the lymphatic vascular system serve as an example of how lack of knowledge about normal and diseased tissues has limited our ability to generate many effective pharmacological therapies. Failure of the lymphatic vascular system has devastating consequences [11]. Lymphedema is the result of inadequate lymphatic function and if not properly managed can lead to debilitating and painful limb swelling, tissue fibrosis, inflammation and increased susceptibility to infection. Lymphedema can be caused by rare genetically-inherited mutations resulting in abnormal development or function of the lymphatic vascular system either at birth, puberty or adulthood [12]. More common causes of lymphedema are due to physical disruption or damage to the lymphatic vasculature, either by surgery, radiation therapy or infection with the mosquito parasites *Wuchereria bancrofti* or *Brugia malayi*. In industrialized countries, the occurrence of lymphedema is rapidly increasing, proportional to the increasing use of surgical and radiation therapies for life-saving cancer treatments. Recent estimates show that one third to nearly one half of women develop lymphedema after breast cancer treatments [13, 14]. In many tropical and sub-tropical countries, parasitic filarial lymphedema or elephantiasis is endemic and affects approximately 120 million individuals worldwide [15]. Regardless of the cause, lymphedema carries with it immense psychological and social sequelae and disability [11].

Current Therapies

Rockson has recently provided a survey of current treatment strategies for the management of lymphatic vascular disease [16]. Currently, the most effective treatment for lymphedema consists of a multifaceted physiotherapeutic approach for improving lymphatic function through application of lymphatic-specific massage techniques, remedial exercise, fitted compression garments and skin care [16]. Although this all-inclusive approach is largely successful among compliant patients [17], the daily treatments and changes in lifestyle can cause undesirable financial, physical and psychological strain for many patients.

Recent surgical approaches have provided limited relief to patients with severe lymphedema. For example, liposuction followed by sustained intense compression can relieve tissue congestion caused by collagen and fat deposition [18, 19]. However, liposuction only provides temporary relief from swelling and does not directly improve lymphatic function. Reconstructive microsurgery through lymphavenous anastomoses (in effect, lymphatic bypass surgery) can provide minimal relief but is largely ineffective [20].

Pharmacological therapies for lymphedema are extremely limited and largely controversial. Coumarin has been shown to reduce lymphedema, but its overall effectiveness and functional mechanism remain controversial [21-23]. Moreover, long term systemic treatment with coumarin is correlated with a high rate of hepatotoxicity [21, 24]. Antioxidents, including selenium and flavonoids, have also been explored as potential therapies, but preliminary investigations into their effectiveness have remained inconclusive.[25] The treatment for parasitic filarial

lymphedema has had better success. Massive drug delivery programs, consisting of dual treatment with diethylcarbamazine and albendazole (anti-parasitic agents), can effectively target and eliminate infecting parasites and reduce the burden of filarial lymphedema in large populations [26]. However, treatment strategies for filariasis do not repair the previously damaged lymphatic vasculature within an individual.

Perhaps the most exciting progress toward pharmacological treatments for lymphedema has come from VEGFC-based therapies. In animal models of lymphedema, VEGFC-based therapies have been demonstrated to promote lymphangiogenesis [27-31]. Even more promising has been the recent discovery that adenovirally-delivered VEGFC can induce the formation of functional collecting lymphatics as well as improve the outcomes of lymph node transplantations in mice [30]. Other growth factors, including VEGFD, VEGFA, fibroblast growth factor-2 and hepatocyte growth factor have also shown exciting promise as therapeutic lymphangiogenic agents in animal models [32, 33]. Nevertheless, it remains clear that the identification of additional drug targets for the modulation of lymphatic growth or permeability is highly desirable.

G-Protein Coupled Receptors as Potential Drug Targets

Because G-protein coupled receptors (GPCRs) are pharmacologically-tractable cell-surface receptors and are widely used for the treatment of human diseases, the identification of potentially useful GPCR targets for the modulation of lymphatic vascular growth or function would represent a major advancement in the field. GPCRs are widely expressed proteins that span the cell membrane 7 times

and respond to a variety of stimuli including peptides, proteins, small organic compounds, lipids, amino acids and cations. Recent estimates predict that humans have approximately 800 GPCRs which can be grouped into five major phylogenetic families: *Rhodopsin, Secretin, Adhesion, Glutamate* and *Frizzled/Taste2* families [34]. A comprehensive review article exploring the diversity between GPCR families and its significance for drug design was recently published by Lagerstrom & Schioth [35]. In summary, GPCRs from every family are highly attractive targets for pharmacological manipulation by either recombinant proteins, small molecule compounds, allosteric ligands or antibodies. There are currently 46 GPCRs that serve as drug targets for the treatment of a multitude of conditions including hypertension, pain, ulcers, allergies, alcoholism, obesity, glaucoma, psychotic disorders and HIV. However, this leaves several hundreds of GPCRs as potential drug targets and among those approximately 150 are still considered orphan receptors (receptors for which the endogenous ligand has yet to be identified) [35]. One major impediment to increasing the repertoire of pharmacologically useful GPCRs is our general lack of knowledge regarding the association of a putative GPCR with a precise physiological function or disease condition. Work presented in this thesis describes the landmark discovery that AM signaling through the Class II GPCR CLR and RAMP2 oligomer represents an essential signaling pathway in lymphatic vascular development and function. Moreover, these findings have the potential to contribute to new therapies for lymphatic vascular disease.

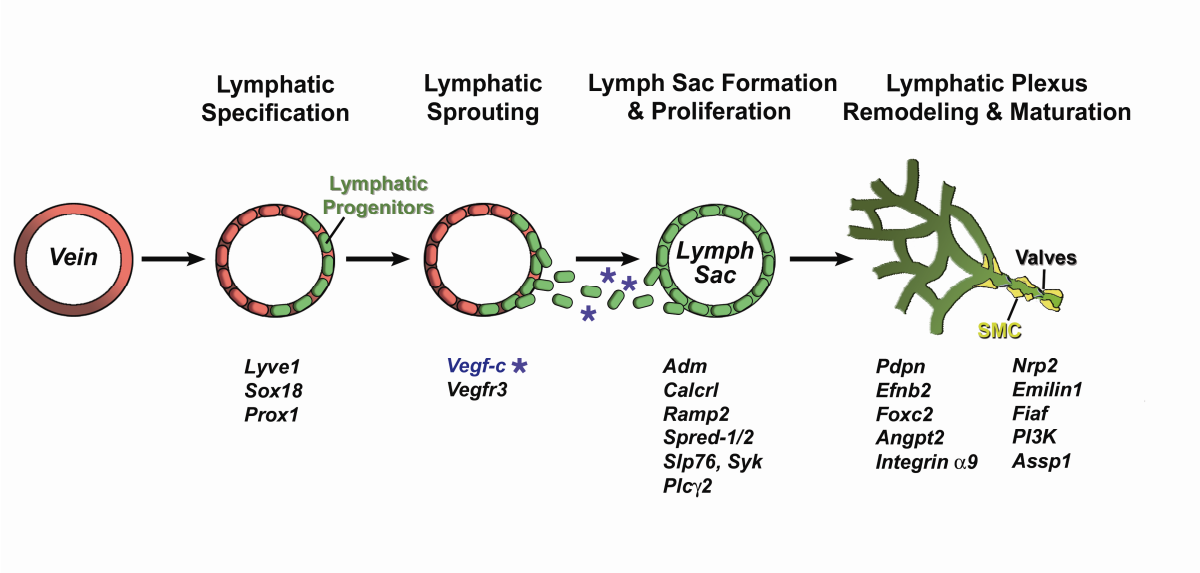

Figure 2.1: The stepwise process of lymphangiogenesis. The development of the lymphatic vasculature begins with specification of lymphatic endothelial cells from venous precursors. The chemoattractant and growth promoting properties of numerous growth factors, including VEGFC, causes lymphatic endothelial cells to sprout and separate from veins to form primitive lymph sacs. Proliferation of lymph sacs leads to the formation of a primary lymphatic plexus which is later remodeled into the mature lymphatic vascular system. Below each step is a list of genes for which a functional role has been demonstrated using genetically engineered mouse models.

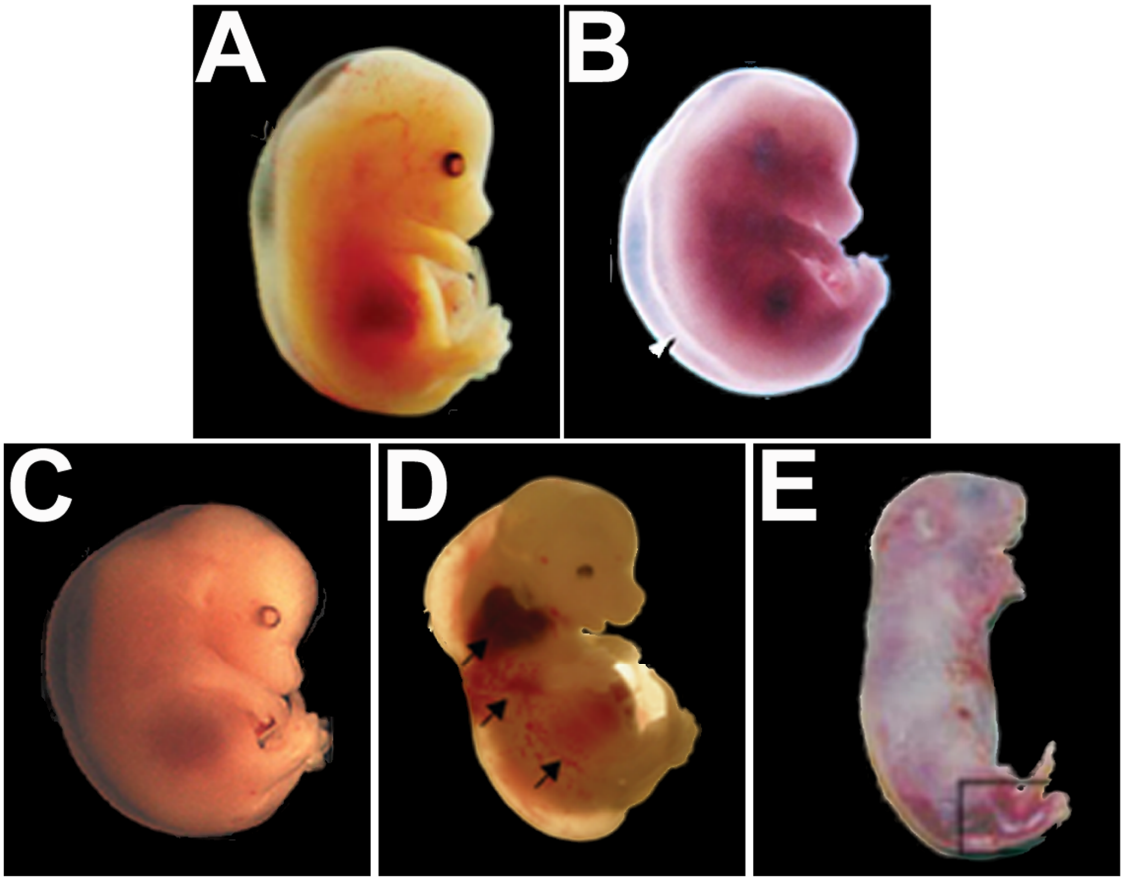

Figure 2.2: Genetic mouse models reveal genes essential for lymphatic vascular development. Generation and characterization of gene targeted knockout mouse models has contributed to the current model of lymphangiogenesis. At mid-gestation, the transcription factors **(A)** Sox18 and **(B)** Prox-1 reprograms venous endothelial cells of the cardinal vein in a polarized manner to become lymphatic precursors [4, 5]. **(C)** VEGFC promotes the migration and differentiation to LECs to form the primary lymph sac [6]. **(D)** Spreds 1 and 2 regulate downstream VEGFC signaling [7]**. (E)** Podoplanin promotes lymphatic vessel maturation and organization [8].

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CHAPTER 3

ADRENOMEDULLIN SIGNALING IS NECESSARY FOR MURINE LYMPHATIC VASCULAR DEVELOPMENT

Abstract

The lymphatic vascular system mediates fluid homeostasis, immune defense and tumor metastasis. Only a handful of genes are known to affect the development of the lymphatic vasculature and even fewer represent therapeutic targets for lymphatic diseases. Adrenomedullin (AM) is a multifunctional peptide vasodilator that transduces its biological effects through the calcitonin receptor-like receptor (*Calcrl*) when the receptor is associated with a novel receptor activity modifying protein (*Ramp2*). Global genetic deletion of each of these genes resulted in a conserved phenotypic series, providing in vivo evidence for the molecular components required for AM signaling during embryonic development. Null mice died at mid-gestation and displayed interstitial lymphedema. A conditional knockout line with loss of *Calcrl* in endothelial cells confirmed an essential role for AM signaling in vascular development. Loss of AM signaling resulted in abnormal jugular lymphatic vessels due to reduction in lymphatic endothelial cell proliferation. Furthermore, AM caused enhanced activation of ERK signaling in lymphatic versus blood endothelial cells, likely due to induction of *Calcrl* gene expression by the lymphatic transcriptional regulator Prox1. Collectively, our studies identify a new class of genes involved in lymphangiogenesis which represent a pharmacologically tractable system for the treatment of lymphedema or inhibition of tumor metastasis.

Introduction

The lymphatic vascular system is an open-ended network of endothelial cell lined vessels that works to transport extravasated tissue fluid, proteins, metabolites and cells from the interstitial space back to the circulatory system via the thoracic duct [1]. Lymphatic capillaries, which lack a basement membrane, are made of overlapping endothelial cells that adhere to the extracellular matrix through fibrillin-rich, pressure-sensing anchoring filaments. Under conditions of interstitial edema, lymphatic endothelial cells stretch apart to collect excess fluid and large proteins. Propulsion of lymph fluid into the larger collecting lymph vessels is largely dependant on the vascular smooth muscle contractions of the upper circulatory trunks. Lymphatic vessels also mediate lipid uptake from the gut and transport white blood cells and antigen-presenting cells to lymphoid organs. Moreover, the lymphatic system plays important roles in pathological conditions such as inflammation, scarring and tumor metastasis [2-5]. Despite its central roles in both normal and disease physiology, our understanding of the development and molecular regulation of the lymphatic vascular system lags far behind that of the parallel blood vascular system [6, 7].

In recent years, advanced genetic engineering techniques have supported the concept that during development lymphatic endothelial cells (LECs) bud from veins to form primary lymph sacs which then proliferate and sprout into the periphery to form lymphatic capillaries and vessels. One of the earliest molecular markers distinguishing venous endothelial cells with a lymphatic bias is the transcription factor prospero-related homeobox 1 (Prox1) [8]. Prox1 is expressed in a polarized

fashion in the venous endothelium and serves as a master switch for establishing LEC competence [9]. Knockout mice for Prox1 show ablated budding and sprouting of primary lymph sacs and die at embryonic day 14.5 (E14.5) with severe edema [10]. Genetic mouse models with loss of VEGFC signaling also develop severe interstitial edema due to failed lymphatic budding and migration [11]. The eventual separation of lymphatic vessels from the venous circulation is largely mediated by the tyrosine kinase Syk and adaptor protein SLP76. Mice with homozygous deletions for the genes encoding these proteins develop arterio-venous shunts and abnormal lymphatic-venous connections [12]. Finally, several genes have been implicated in the later stages of lymphatic vessel patterning and postnatal maturation, including podoplanin [13], neuropillin2 [14], FoxC2 [15] and angiopoetin-2 [16]. While a few of these gene products, like VEGFC and angiopoetins, show promise as potential targets for the stimulation, inhibition or modulation of lymphangiogenesis and lymphatic function in lymphedematous tissues or tumors of adult animals [17], there remains a crucial need to identify additional genetic and pharmacologically-tractable targets that regulate the development and function of the lymphatic vascular system [1, 18].

Adrenomedullin (AM) is a multifunctional 52-amino acid peptide vasodilator that is pathologically elevated in a variety of tumors [19] and cardiovascular conditions [20]. In addition to its function as a potent vasodilator, AM is also recognized as an important vascular factor involved in angiogenesis and endothelial cell proliferation. AM treatment potently induces the proliferation, migration and capillary tube formation of cultured human umbilical vein endothelial cells

(HUVECs) and directly promotes endothelial cell growth and survival through activation of MAPK/ERK downstream signaling pathways [21-26]. Similar results have been recapitulated in vivo through the use of a hind-limb ischemia model [27] and a differentiated ES cell culture model [28] in which AM treatment promotes an angiogenic response. Taken together, these data demonstrate that AM signaling in the blood vasculature plays an important role in vessel development, remodeling and function, however its role in the lymphatic vascular system has yet to be addressed.

AM binds and signals through a G-protein coupled receptor, calcitonin receptor-like receptor (CLR= protein; *Calcrl*=gene). The ligand binding affinity of CLR (and other class II GPCRs) can be changed via receptor interaction with a class of single-pass transmembrane proteins called receptor activity modifying proteins (RAMPs) [29]. Since the AM receptor and the three mammalian RAMPs are highly expressed in the vasculature, this paradigm of cell signaling is being explored pharmaceutically for the potential treatment of conditions like migraine [30], pulmonary hypertension [31] and other cardiovascular disorders [32] and for the modulation of cancer progression [33]. However, determining the functional significance of AM signaling on the vasculature during embryogenesis and adulthood remains an area of intense investigation which we here address with genetically engineered animals that lack either *AM*, *Calcrl* or *Ramp2* globally or that lack *Calcrl* specifically in endothelial cells.

Experimental Methods

Animals

The generation and characterization of mice with targeted deletions of the *AM*, *Calcrl* and *Ramp2* genes has been described previously [34-36]. Gene targeting for the *AM*, *Calcrl* and *Ramp2* genes was performed with targeting vectors and ES cells of the SvEv129/6-TC1 background. Chimeric male mice were then bred to SvEv129/6-TC1 female mice in order to establish completely isogenic colonies. In the case of *Ramp2*, we have previously reported that on the SvEv129/6-TC1 isogenic background there are profound fertility defects [34]. These fertility defects are rescued on an F1 (SvEv129/6-TC1:C57Bl6) background and so animals derived from F1 intercrosses were used for analysis in the current study.

To generate *Calcrl*$^{Flox/Flox}$ mice, a *Calcrl* floxed targeting vector was generated using a 129S6/SvEv phage clone (previously described[35]) containing exons 3 through 9 of the *Calcrl* gene. LoxP sites were inserted outside of exons 5 and 6. The long arm of homology included exons 7, 8 and 9, while the short arm included exons 3 and 4. Standard gene targeting methods were utilized to generate embryonic stem cells and mice with the floxed *Calcrl* allele. Briefly, E14 ES cells were electroporated with the linearized targeting vector shown in Figure 2A. After positive (G418) and negative (ganciclovir) selection 7 positive ES cell clones were identified from 96 selected clones. Primers p1 and p4 were used for PCR-based screening of ES cells and primers p2, p3 and p4 were used for genotyping of mice (**Figure 3.2A and Appendix: Supplemental Figure 6**). Male chimeric mice that transmitted the targeted allele were bred to C57BL/6J females and homozygous

Calcrl$^{Flox/Flox}$ were generated. Targeting was confirmed by Southern blotting using a probe indicated in Figure 2A.

B6.Xcg-Tg (tek-cre)12Flv/J (*Tie2Cre*) mice were obtained from Jackson Laboratories. Endothelial specific *Calcrl* null embryos were generated by crossing *Calcr/*$^{+/-}$ female mice to *Tie2Cre*+ male mice. The resulting *Calcrl*$^{+/-}$*;Tie2Cre*+ animals were then bred to *Calcrl*$^{Flox/Flox}$ resulting in experimental animals for this study.

For all studies, littermate controls were utilized. The day of the vaginal plug was considered embryonic day 0.5 (E0.5). All experiments were approved by the Institutional Animal Care and Use Committee of The University of North Carolina at Chapel Hill.

Antibodies and Growth Factors

The following primary antibodies were used in this study: rat anti-mouse CD31(PECAM) monoclonal (BD Biosciences), rabbit anti-Prox1 (Chemicon), monoclonal anti-α smooth muscle actin clone 1A4 (Sigma), goat anti-mouse VEGFR3 (R&D Systems), Mouse anti-Phospho-ERK1/2 (p44/42) antibody (Thr202/Tyr204) and rabbit anti-ERK antibody (Cell Signaling Technology). F-actin was stained with FITC-Phalloidin (Sigma) and nuclei were stained with Hoechst 33258 (Sigma).

Human AM and human AM_{22-52} were purchased from American Peptide, PD98059 from Sigma-Aldrich, and recombinant human VEGFA from Pierce Biotechnology.

In Vivo Proliferation Assay

Pregnant females were injected intraperitoneally with 100µg/g body weight BrdU (Sigma, St. Louis). After 1 hour, mice were euthanized and embryos recovered for immunohistochemistry as described in **Appendix: Supplemental Methods**. BrdU positive cells were detected with the BrdU staining kit (Zymed) followed by staining in Mayer's hematoxylin. Percent proliferative cells was determined by blinded quantitation of BrdU positive cells in the lymphatic or vascular endothelium divided by total hematoxylin stained nuclei. Data was analyzed using the two-tailed Student's t-test assuming unequal variance.

High Resolution, 3-D Optical Projection Tomography (OPT)

Whole mount immunofluorescent optical projection tomography was performed by BIOPTONICS (Edinburg, GB) following manufacturer's protocol. Briefly, E13.5 (PECAM) and E14.5 (VEGFR3) embryos were fixed in DENTS fixative overnight, permeablized in acetone, and washed with PBST. Embryos were incubated in primary antibodies and 5% Goat serum, 1% BSA and 1% DMSO in PBST for 8-10 days. Specimens were washed in multiple changes of PBST for up to 4 days, and secondary antibodies in PBST were added. After 8 days, embryos were washed in PBST followed by PBS and then imaged by BIOPTONICS OPT scanner 3001 (MRC Technology).

Cell Culture

HUVECs were obtained from Lonza (Walkersville, MD) and maintained in endothelial cell medium provided by the supplier and used at passages 3-7. HMVEC-dLys (LECs), product number #CC-2810, were purchased from Lonza and maintained in endothelial cell medium provided by the supplier and used at passages 3-7. Culture media was changed every 2-3 days and cells were passed when they achieved 90% confluency.

In Vitro Proliferation Assay

Cell proliferation was measured by modified MTS assay as previously described [37]. Briefly, LECs and HUVECs were plated at 8 x 10^4 cells/ml on a 96-well plate. After 24h in normal growth conditions, media was changed to basic medium containing 0.5% FBS for 24h. Cells were then treated with appropriate concentration of peptide (AM or VEGFA in replicates of 5) under low serum conditions for 48h at which point, 20 µl of MTS tetrazolium compound (CellTiter 96Aquesous, Promega) was added to each well and incubation was continued for 3 hours. Absorbance at 490nm was recorded and background was corrected by subtracting the average absorbance observed in wells containing no cells. Percent proliferation defined as the corrected absorbance in treated samples divided by corrected absorbance of untreated samples.

For total cell count determinations, LECs and HUVECs were plated at 2 x 10^5 cells/ml in each well of 6-well plates. After 24h in normal growth conditions, media was changed to basic medium containing 0.5% FBS for 24h. Cells were then

treated with 10nm of AM peptide under low serum conditions for 48h. At 48h, the cells were lifted off the plate by application of 0.25% Trypsin (Gibco) and counted visually with a hemocytometer. Percent proliferation was a measure of treated samples divided by untreated samples. Experiments were performed in duplicate and data is representative of 3 separate experiments.

Prox1 Transfections

For electroporation of HUVECs and LECs the plasmid pFlag, either empty or containing full length hProx1 [38], a kind gift from Dr. Eckard Truter, Karolinski Institute, Sweden, were electroporated in triplicate. 1 X 10^6 HUVECs or LECs were electroporated using the HUVEC Nucleofector kit (Amaxa). 24 hours after transfection RNA was generated for quantitative RT-PCR as described above.

Tissue Preparation, Immunohistochemistry and Microscopy

Tissues or cells, plated on glass coverslips, were fixed in 4% paraformaldehyde in PBS overnight. For immunofluorescent staining, tissues were then cryoprotected with 30% sucrose in PBS overnight, embedded in OCT (Tissue-Tek) and cryosectioned at 6-8µm. Sections or cells were rehydrated in PBS, permeablized with 0.4% Triton-X100 in PBS, blocked with 4% BSA in PBS and incubated in primary antibody overnight. After washing, fluorescently labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were incubated for 2 hours at room temperature, sections were washed and mounted for imaging. EGFP was imaged immediately after permeablization. Images were acquired on a

Nikon E800 microscope with a Hammamatsu ORCA-ER CCD camera using Metamorph Software (Molecluar Devices) and processed in Photoshop8.0 (Adobe). For hematoxylin and eosin staining, fixed tissues were put through an ethanol gradient, embedded in paraffin and sectioned at 10µm. Sections were deparaffinized and stained with Mayer's hematoxylin and eosin following standard protocols.

Quantitative RT-PCR

Lymphatic vessels were isolated from the hind limbs of adult SvEv129/6 mice. Briefly, the hind footpads of anesthetized mice were injected with 3% Chicago sky blue in Tyrode's Buffer. After incorporation, mice were euthanized and lymphatic vessels labeled with blue dye were excised under a dissecting microscope. RNA was isolated using the RNeasy Micro kit (Qiagen, Valencia, CA) with additional DNAse treatment (Promega). RNA from embryos and cultured cells was isolated using the RNeasy Mini kit. cDNA was then generated using the iScript Select cDNA synthesis kit (Bio-Rad, Hurcules, CA).

Quantitative PCR was performed on the Stratagene Mx-3000p machine (La Jolla, CA) using ABgene ROX master mix (Rochester, NY) and rodent GAPDH control reagents (Applied Biosystems, Foster City, CA). Relative levels of gene expression were determined using the comparative quantitation ($\Delta\Delta$CT) method with MxPro software (Stratagene). Sequences for the mouse *AM*, *Calcrl* and *Ramp2* primers and probes have been published [35, 36, 39] and all other primer and probe sequences are provided in the Appendix. All assays were repeated at least twice,

and all samples were run in triplicate. Results were analyzed using the two-tailed Student's t-test assuming unequal variance.

Interstitial fluid analysis

To collect interstitial fluid from homozygous null embryos, *Ramp2*$^{-/-}$ E14.5 embryos were dissected in PBS. A 30.5 gauge needle was inserted under the edematous skin and fluid was slowly drained. Rat lymph was collected directly from the thoracic duct of an adult Sprague-Dawley rat and mouse serum was collected by tail bleed from adult SvEv129/6 mice. Samples were centrifuged at 30,000x g and supernatants were loaded onto 8-12% gradient polyacrlymide tris-glycine gels. Gels were stained with coomassie blue 250 (Sigma).

Quantification of Lymph Sac Area

Hematoxylin and eosin stained transverse sections of the jugular region of *AM, Calcrl and Ramp2* null embryos were imaged on a Leica MZ16 dissecting microscope. Sections containing jugular lymph sacs that were matched for the same anteroposterior level were blindly measured using ImageJ software (NIH, USA). In order to normalize for section variability, the area of the lymph sac (defined as the area within the perimeter) was divided by the area of the adjacent jugular vein.

Transmission Electron Microscopy

Embryos were collected and fixed in 2% formaldehyde/2.5% glutaraldehyde in 0.15M sodium phosphate buffer, pH 7.4, overnight at 4˚C. Following several washes in sodium phosphate buffer, the samples were post-fixed for 1 hour in potassium ferrocyanide-reduced osmium, dehydrated through a graded series of ethanol and embedded in PolyBed 812 epoxy resin (Polysciences, Warrington, PA). Using a diamond knife, 1 μm cross-sections were cut, stained with 1% toluidine blue and examined by light microscopy to isolate areas containing jugular lymph sacs. Ultrathin sections were cut with a diamond knife (70-80 nm thickness), mounted on 200 mesh copper grids and stained with 4% aqueous uranyl acetate for 15 minutes followed by Reynolds' lead citrate for 8 minutes. The sections were observed using a LEO EM-910 transmission electron microscope (LEO Electron Microscopy, Inc., Thornwood, NY), accelerating voltage of 80 kV, and micrographs were taken using a Gatan Orius SC 1000 CCD Camera (Gatan, Inc., Pleasanton, CA).

In Cell Western

$8x10^4$ cells/ml were cultured under normal growth conditions and after 24 hours media was changed to basic medium containing 0.5% FBS for 24 hours. Cells were then treated with 10nm AM for indicated times. At the end of incubation, the medium was removed and the cells were fixed, permeabilized, blocked, and treated with the appropriate antibodies per manufacturer's instructions (LI-COR). The primary antibodies, anti-phosph-ERK1/2 and anti-ERK1/2, were used at

dilutions 1:100 and 1:25, respectively, and detected with goat-anti-mouse IR680 and goat-anti-rabbit IR800 each at 1:5000 dilutions. The plates were scanned with the Odyssey infrared scanner, data was obtained using scanner software and total area under the curve was determined by Gaussian best-fit analysis.

Western Blotting

LECs were treated with 10nm AM for indicated time points, washed three times with PBS, lysed in 100µl of sample buffer, boiled for 10 min, loaded onto a 10% polyacrylimide gel and resolved by SDS-PAGE. Protein was then transferred to a polyvinylidene diflouride (PVDF) membrane which was subsequently blocked in 5% non-fat dry milk overnight at 4 degrees. The primary antibodies were incubated overnight at 4 degrees at 1 mg/ml and detected with secondary antibodies as described for the In Cell Western above. The blot was imaged with the Odyssey infrared scanner and data was obtained using scanner software.

Results

The RAMP2-CLR complex is the functional AM receptor during embryonic development.

AM$^{-/-}$, *Calcrl*$^{-/-}$ and *Ramp2*$^{-/-}$ embryos failed to survive and developed severe interstitial edema without hemorrhage by mid-gestation (**Figure 3.1A,D,G**). Histology of the *AM*$^{-/-}$, *Calcrl*$^{-/-}$ and *Ramp2*$^{-/-}$ embryos confirms the presence of extreme interstitial edema and the notable absence of hemorrhagic plaques (**Figure 3.1B,C;E,F;H,I**). The onset of edema between the different lines was temporally

staggered by approximately 24 hours, after which all null embryos were found dead 1-2 days later. The delay in phenotypic onset in *Ramp2*$^{-/-}$ embryos suggests that some compensatory signaling for AM, perhaps through a RAMP3-CLR complex, may still exist during embryonic development. However, our previous observations that RAMP3 null mice survive to adulthood with few phenotypic defects [34] demonstrates that RAMP3 is unable to fully replenish RAMP2-mediated AM signaling in vivo. Moreover, we found that *Ramp2*$^{-/-}$ embryos displayed remarkably similar developmental defects to those we have previously described for *AM*$^{-/-}$ and *Calcrl*$^{-/-}$ mice [35, 36] **(Appendix: Supplementary Figure 1)**. Therefore, this conserved phenotypic series from three independent knockout mouse lines provides compelling genetic evidence to define CLR and RAMP2 as the receptor components required for AM signaling during embryogenesis.

Absence of AM signaling results in generalized interstitial edema and embryonic lethality.

Generalized edema and mid-gestation lethality can be caused by a multitude of developmental defects during embryogenesis. To determine whether the loss of AM signaling in vascular endothelial cells could account for the embryonic edema and/or lethality of global *AM*$^{-/-}$, *Calcrl*$^{-/-}$ and *Ramp2*$^{-/-}$ embryos, we generated mice with a conditional allele for the *Calcrl* gene. Using the Cre-LoxP gene targeting strategy, we generated homozygous “floxed” mice for the *Calcrl* gene and confirmed correct gene targeting by Southern blot and genomic PCR (**Figure 3.2A-C**). Importantly, our gene targeting approach ensured that the loxP sites flanked the

same genomic regions as those deleted in the original *Calcrl* knockout mouse [35], thereby eliminating the possibility of generating allelic phenotypes. Homozygous floxed *Calcrl* mice (*Calcrl*$^{Flox/Flox}$) appeared normal, bred well and had levels of *Calcrl* gene expression that were indistinguishable from wildtype animals (**Figure 3.2D**).

As shown in the schematic of **Figure 3.2E**, we bred the *Calcrl*$^{Flox/Flox}$ mice to mice that were heterozygous for the *Calcrl* gene knockout and positive for expression of Cre recombinase in endothelial cells via expression of a *Tie2-Cre* transgene [40]. By breeding onto a *Calcrl*$^{+/-}$ genetic background we ensured robust excision of a single floxed allele and thereby reduced the incidence of mosaic cellular excision. Importantly, *Calcrl*$^{+/-}$ mice are born at the expected Mendelian ratio and survive to adulthood with few phenotypic defects [34]. The resulting mice from the Cre-LoxP breeding strategy, *Calcrl*$^{LoxP/-}$*;Tie2Cre*+, were therefore global heterozygotes for *Calcrl* and were lacking *Calcrl* expression specifically in endothelial cells as early as E10.5. We noticed a slight reduction in the Mendelian distribution of *Calcrl*$^{FloxP/-}$*;Tie2Cre*- mice (**Figure 3.2F**) which is consistent with the leakiness of the *Tie2-Cre* transgene in the female germline leading to global *Calcrl* excision and embryonic lethality [40]. Consistently, the *Calcrl*$^{LoxP/-}$*;Tie2Cre*+ mice were embryonic lethal and developed extreme interstitial edema without hemorrhage, sometimes as early as E13.5 (**Figure 3.2F,G**). However, in contrast to the global *Calcrl*$^{-/-}$ mice which die by E13.5, the onset of edema and eventual demise of the *Calcrl*$^{LoxP/-}$*;Tie2Cre*+ mice was often substantially delayed to ~E16.5, three to four days later than the global *Calcrl*$^{-/-}$ mice. Thus, we conclude that endothelial-specific deletion of *Calcrl* prolonged the survival of AM signaling mutants during

development, but nevertheless resulted in similar generalized edema without hemorrhage compared to the global *Calcrl* knockout mice. These data demonstrate that the cellular basis of the embryonic edema and eventual lethality in AM signaling mutant mice is due to loss of AM signaling in endothelial cells.

AM signaling is activated during lymphangiogenesis.

Despite the well-established role for AM in maintaining vascular permeability [41, 42], we very rarely observed blood hemorrhage and only occasionally in embryos that were visibly deteriorated or near death. For example, out of 72 wildtype embryos, 7 (9.7%) exhibited some form of visible hemorrhage and only 4 out of 56 *Ramp2*$^{-/-}$ littermates (7.1%) showed evidence of hemorrhage. Thus, the remarkable lack of embryonic hemorrhage in the *AM*$^{-/-}$, *Calcrl*$^{-/-}$ and *Ramp2*$^{-/-}$ embryos suggested to us that the developing blood vasculature remained largely intact during embryonic development. Moreover, presence of high MW proteins in the interstitial fluid confirmed that the null mice suffered from embryonic lymphedema as opposed to osmotic imbalance (**Appendix: Supplementary Figure 2**). Thus, based on the absence of blood hemorrhage, the presence of embryonic lymphedema and the fact that the edematous phenotype of AM signaling null mice more closely resembled that of other mouse models with defects in lymphangiogenesis [10, 11, 14] rather than defects in blood vascular permeability [43-46] we hypothesized that AM signaling might be principally important for orchestrating normal lymphangiogenesis.

First, we demonstrated that *AM*, *Calcrl* and *Ramp2* are expressed in adult lymphatic vessels at levels similar to those observed in whole embryonic RNA extracts (**Figure 3.3A**). To determine if *AM*, was also expressed in lymphatic vessels during embryonic development, we made use of an enhanced green fluorescent protein (EGFP) that was inserted at the initiator methionine of the endogenous *AM* gene by homologous recombination and serves as a biological marker of *AM* gene expression in $AM^{+/-}$ and $AM^{-/-}$ mice [36]. As shown in **Figure 3.3B**, *AM* was expressed in endothelial cells lining the jugular vein and budding lymph sac at E12.5, which correlates with the earliest stages of lymphatic vascular development. Interestingly, we noticed that the pattern of *AM* expression in the jugular vein was often polarized towards the developing lymph sac, similar in pattern to that of the master transcriptional regulator of lymphatic specification, Prox1 [9]. We therefore conclude that the expression of genes required for AM signaling is robust in adult and developing lymphatic endothelial cells (LECs) and is similar in pattern to that of Prox1 during embryogenesis.

We next performed immunohistochemistry using LEC markers to determine if AM signaling was required for the initial formation of jugular lymph sacs. Distinct lymph sacs were present in wildtype, $Ramp2^{-/-}$ and $AM^{-/-}$ embryos by E13.5 (**Figure 3.4A-C**) and stained positively for PECAM and the lymphatic specific makers VEGFR3 and Prox1, indicating normal lymphatic differentiation from venous vasculature. Similar results were observed in $Calcrl^{-/-}$ embryos (data not shown). We also identified distinct VEGFR3-positive vessels in the skin of $Ramp2^{-/-}$ embryos (**Figure 3.4D,E**), indicating that the development of the dermal lymphatic vasculature

remains intact in AM signaling mutant mice. From these findings, we conclude that AM signaling is not required for the early stages of lymphangiogenesis, including differentiation and migration of LECs and the formation of primary lymph sacs and dermal lymphatic vessels.

AM signaling regulates lymphatic endothelial proliferation.

Although immunohistochemistry revealed the presence of lymph sacs in the AM signaling null mouse models, we noticed from histological sections that the jugular lymph sacs appeared strikingly smaller than those of litter-matched wildtype embryos (**Figure 3.5A-D**). To confirm the apparently hypoplastic lymph sacs, we used computerized morphometry to calculate lymph sac area and found that all null mice showed significantly reduced lymph sac area compared to wildtype embryos at E13.5 (**Figure 3.5E-G**). Thus, despite excessive interstitial lymphedema (which would normally cause lymphatic vessel distension), the lymph sacs of the AM signaling null embryos remained significantly smaller than wildtype lymph sacs, suggesting either a functional failure of the early lymphatics to take up extravasated fluid or an abnormality in LEC growth and proliferation.

To determine whether an ultrastructural defect in LECs could lead to a functional failure of lymphatic vessels during development, we performed transmission electron microscopy of *Ramp2*$^{-/-}$ and wildtype mice at E14.5. LECs of the jugular lymphatic vessels in wildtype mice displayed typical features including overlapping cell-cell contacts (**Figure 3.6A**, arrowheads), electron-dense tight junctions (**Figure 3.6B,C**, arrows) and anchoring filaments (**Figure 3.6A**). Wildtype

lymphatic vessels at this stage of development also lacked a basement membrane and lymphatic smooth muscle cells (**see also Appendix: Supplemental Figure 3**), thus precluding the possibility that failure of smooth muscle recruitment could account for the lymphatic phenotypes observed in the AM signaling null mice. In *Ramp2*$^{-/-}$ mice the overlapping cell-cell contacts, tight junctions and anchoring filaments remained intact (**Figure 3.6D, E**). Occasionally, necrotic LECs were observed in *Ramp2*$^{-/-}$ mice (**Figure 3.6F**), but the tight junctions nevertheless remained intact. Consistently, we found that *Ramp2*$^{-/-}$ LECs appeared markedly thinner than wildtype LECs. Based on these data, we found no substantial evidence to suggest that the lymphatic vasculature of the *Ramp2*$^{-/-}$ mice would fail to function due to ultrastructral abnormalities.

To determine whether the smaller lymph sacs of AM signaling mutant mice could be due to abnormal endothelial cell proliferation, we used a BrdU incorporation assay to count proliferating cells in both lymph sacs and adjoining jugular veins. In *AM*$^{-/-}$, *Ramp2*$^{-/-}$ and *Calcrl*$^{-/-}$ embryos, there were significantly fewer proliferative endothelial cells in the lymph sacs, while the proliferation of the venous endothelial cells, although somewhat reduced in the null mice, never reached statistical significance (**Figure 3.7A,B,C**). Taken together these results demonstrate that lack of AM signaling during embryonic development leads to a specific reduction of lymphatic, but not venous, endothelial cell proliferation.

To confirm that loss of AM signaling preferentially affected the development of the lymphatic versus blood vascular system, we used whole mount immunohistochemistry for VEGFR3 or PECAM followed by 3-dimensional optical

projection tomography [47] in *Ramp2*$^{-/-}$ mice. While we found no obvious differences in the morphological development of the blood vascular system between wildtype and knockout mice (**Figure 3.7D,E**), we did observe extraordinary differences in the jugular lymphatic trunks (**Figure 3.7F,G**, large white arrows). While wildtype mice had large, well-formed jugular lymphatic trunks, the same vessels were essentially absent in the *Ramp2*$^{-/-}$ littermates (**Figure 3.7 and Appendix: Supplemental Movies 4 & 5**). Notably, the retroperitoneal lymph vessel (which develops into the thoracic duct) and the dermal lymphatic vessels of *Ramp2*$^{-/-}$ embryos appeared indistinguishable from those of wildtype littermates (**Figure 3.7**, small yellow arrows and **Figure 3.4D,E**). Therefore, we conclude that AM signaling is preferentially required for the normal proliferation and morphological integrity of the jugular lymphatic vessels during the later stages of lymphangiogenesis.

AM signaling is enhanced in lymphatic endothelial cells.

To determine the downstream signaling cascades that are associated with the enhanced activity of AM signaling in LEC proliferation, we made use of two distinct endothelial cell lines; human umbilical vein endothelial cells (HUVECs) and human dermal lymphatic microvascular endothelial cells (HMVEC-dLy, herein referred to as LECs). Histology and immunocytochemistry of the LEC cell line demonstrated different cellular morphology and the expression of Prox1 compared to HUVECs, which are consistent with the characterization of these cells as lymphatic endothelial cells (**Figure 3.8A**). Stimulation of HUVECs and LECs with vascular endothelial cell growth factor A (VEGFA) resulted in an expected and dose-dependant increase in

cellular proliferation that did not differ significantly between the two cell lines (**Figure 3.8B**). In contrast, while AM elicited a modest yet significant increase in HUVEC proliferation, the proliferative effects of AM on LECs were substantially more robust and significantly different than the effects of AM on HUVEC proliferation at 10nM (**Figure 3.8C**). Direct cell count determinations verified that LECs treated with 10nM AM for 48 hours showed two-fold more proliferation than similarly treated HUVECs (81% vs. 37% more than control, $p<0.01$, data not shown).

To determine the downstream signaling pathways associated with the effects of AM signaling on LEC proliferation, we quantitatively assessed the activation of ERK in HUVECs and LECs that were treated with 10nM AM. Using a Gaussian fit-curve analysis, we found that AM stimulation of LECs resulted in approximately twice the overall levels of phosphorylated ERK over a 30 minute time course compared to HUVECs (**Figure 3.8D**). Inhibition of AM-mediated ERK phosphorylation by a RAMP2-specific AM inhibitor, AM22-52 [48], and by the MAPK inhibitor, PD98057, confirmed that ERK activation by AM signaling in LECs is mediated through a RAMP2-CLR receptor complex (**Figure 3.8E**). Taken together, these comparative studies in cultured endothelial cells demonstrate that AM signaling, through a RAMP2-CLR receptor complex, has a preferential and enhanced effect on ERK-mediated LEC proliferation compared to HUVECs.

Several comparative transcriptional profiling studies have shown enhanced expression of AM signaling genes in lymphatic versus blood endothelial cells [49, 50] and lymphedematous versus normal vasculature [51]. Therefore, we hypothesized that the enhanced sensitivity of LECs to AM signaling could be due to preferential

up-regulation of the receptor components required for AM signaling. Quantitative RT-PCR for *AM*, *Calcrl* and *Ramp2* confirmed our assumptions and showed approximately 4 times higher levels of gene expression in cultured LECs compared to HUVECs (**Figure 3.9A**). Because we had previously observed that the expression of AM in vivo closely matched the pattern of Prox1 expression in the developing lymphatic sacs of E13.5 embryos (**Figure 3.3B**), we next wanted to determine whether the AM receptor, *Calcrl*, could be a downstream target gene of Prox1. Therefore, we transfected a hProx1 expression plasmid into cultured LECs and measured mRNA production of enodgenous *LYVE* and *Calcrl*. Consistent with previous studies showing that Prox1 is not required for the lymphatic-specific expression of LYVE [10], over-expression of Prox1 in LECs did not induce the expression of LYVE (**Figure 3.9B**). In contrast, over-expression of Prox1 resulted in a 3-fold increase in endogenous *Calcrl* expression levels compared to empty vector control transfections (**Figure 3.9C**). We therefore conclude that the expression of genes required for AM signaling is preferentially robust in LECs compared to HUVECs and furthermore inducible by Prox1.

Discussion

We have performed a comparative phenotypic characterization of three independent gene knockout models for genes that encode proteins purported to be involved in signaling for AM peptide. The remarkable conservation in phenotypes between knockout mice with targeted loss of either AM peptide, the GPCR receptor CLR or its modifying protein, RAMP2 provides compelling genetic evidence to define

the receptor components required for AM signaling during embryogenesis. The paradigm of RAMP-GPCR signaling offers the exciting opportunity to generate pharmacological compounds that specifically interact at the RAMP-GPCR interface for modulating the activity of several peptide agonists. As an example, the small molecule compound BIBN4096BS [52] specifically binds to RAMP1-CLR complexes to antagonize CGRP activity and is currently in clinical trials for treatment of migraine [53]. Our conserved phenotypic series of null mouse models suggest that compounds specific to the RAMP2-CLR interface would provide the best pharmacological target for modulation of AM activity in vivo.

AM is a secreted peptide vasodilator that is highly expressed in blood endothelial cells and induced by hypoxia to promote angiogenesis and inhibit vascular permeability [31, 54]. Here we show that AM and its receptors are more highly expressed in LECs under the control of the lymphatic-specific transcriptional regulator Prox1. Therefore, the enhanced level of CLR receptor expression disposes LECs to a more robust AM response than blood endothelial cells (**Figure 3.10**). As a consequence, global genetic loss of either *AM*, *Calcrl* or *Ramp2* causes marked and preferential reduction in the proliferation of lymphatic endothelial cells of the jugular lymphatic vessels. Our current explanation for the cause of edema in these mouse models is that lack of lymphatic proliferative signals during lymphangiogenesis results in smaller, lower capacity jugular lymphatic vessels that are unable to accommodate the normal uptake of extravasated fluid and thus exacerbates massive interstitial edema. It is also worth emphasizing that the precise cause of embryonic lethality has yet to be determined.

A multitude of developmental defects during embryogenesis can lead to edema and mid-gestation lethality. For example, we cannot at this time exclude the possibility that global loss of AM signaling might also cause excessive permeability of the blood vasculature, contributing to the overall hydrops phenotype. In this regard, our data suggest that global loss of either *AM*, *Calcrl* or *Ramp2* results in thin vascular smooth muscle coverage of large blood vessels [35, 36] (**Appendix: Supplementary Figure 1E-F**). However, based on the absence of hemorrhage and the remarkable similarity of our phenotype to other genetically engineered mouse models with defects in lymphangiogenensis [10, 11, 14] (rather than mouse models with leaky blood vasculature or thin vascular smooth muscle walls [43-46]), we contend that the major contribution to the overall hydrops phenotype in AM signaling null mice comes from the loss of gene function in the lymphatic vasculature. We have also shown that global loss of either *AM*, *Calcrl* or *Ramp2* results in smaller hearts with thin compact zones and ventricular trabeculae [35, 36] (**Appendix: Supplementary Figure 1 A-D**), and heart failure can sometimes contribute to embryonic edema. However, since the AM signaling null mice do not suffer from hepatic congestion or bradycardia and since the edema is characteristically “generalized interstitial edema” (as opposed to pericardial effusion which most often accompanies embryonic heart failure) we feel it is unlikely that heart failure is the principle cause of embryonic edema.

Our generation and characterization of mice with conditional loss of *Calcrl* in vascular endothelial cells further supports our interpretation for a principal and essential role for AM signaling in endothelial cells during embryonic development.

Since expression of the *Tie2-Cre* transgene precedes the onset of lymphangiogenesis in embryonic veins and since the *Tie2* promoter is active in developing lymphatic endothelial cells [55], it is likely that excision of the *Calcrl* gene also occurs robustly in the developing LECs of the *Calcrl*$^{LoxP/-}$*;Tie2Cre*+ mice. However, the *Tie2-Cre* transgene is also expressed in cells of the hematopoietic lineage, which have modest expression of AM signaling components [56, 57] and have been previously demonstrated to contribute to lymphatic vascular development [58]. Therefore, we cannot exclude the possibility that loss of AM signaling in the hematopoietic lineage also contributes to the overall phenotype of the *Calcrl*$^{LoxP/-}$*;Tie2Cre*+ mice. Similarly, we cannot exclude the possibility that loss of AM signaling in cardiac structures derived from *Tie2-Cre* expressing progenitors (for example, mesenchymal cells of the atrioventricular canal and the proximal outflow tract) may contribute to the phenotype. Future studies with conditional deletion of the *Calcrl* gene exclusively in BECs, LECs or hematopoietic cells will help address these caveats, but suitable Cre transgenic mouse lines with exclusive expression of Cre in these cell types must first be generated.

Hypoplastic growth of the lymphatic vasculature during development has also been described in mice that lack *neuropillin2* [14], a co-receptor for the lymphangiogenic growth factor VEGFC. One assumption that can be made from these apparently similar phenotypes is that VEGFC and AM signaling may interact simultaneously to control the proliferation of the developing lymphatic vessels during embryogenesis. However, in contrast to the AM signaling null phenotype, loss of *neuropillin2* selectively and transiently compromised the proliferation of smaller

lymphatic vessels and capillaries, but not jugular lymphatic vessels, during development. Together, these results demonstrate that the development of small and large lymphatic vessel populations is under the control of different lymphangiogenic factors and that these growth factors may work autonomously in lymphatic vessels that arise from different progenitor populations.

Our current study and those of others [21-26] show that AM signaling directly promotes endothelial cell growth and survival through activation of MAPK/ERK downstream signaling pathways. In contrast, the Sprouty/Spred family of proteins mediates an important negative regulation of growth factor- and cytokine-induced MAPK/ERK activation. Recently, Taniguchi *et al* showed that mice with double genetic deletion of Spred 1 and 2 develop marked edema and dilated, blood-filled lymphatic vessels [59]. While the number of blood vessels and blood endothelial cells in Spred-1/2 deficient embryos did not differ from wildtype, the authors found a significant increase in the number of LECs and lymphatic vessels which was due to abnormal loss of VEGFC/VEGFR-3 inhibition and subsequent higher ERK activation. The phenotype of *hyperplastic* lymphatic vascular growth by *enhanced* ERK activation in Spred-1/2 knockout mice stands in notable contrast to our AM signaling null phenotype of *hypolastic* lymphatic vascular growth caused by *decreased* ERK activation in LECs. Taken together, the comparative findings from the two studies are entirely consistent with the notion that the tight control of ERK activation by vascular growth factors is essential for the controlled proliferation of LECs during development.

Failure or insufficient function of the lymphatic vascular system results in disfiguring, disabling and sometimes life-threatening swelling of the limbs, called lymphedema [60] which most often occurs as a result of a parasitic filarial infection or secondary to surgical removal of lymph nodes, radiation therapy or infection [2]. Recent studies have also brought the lymphatic system to the forefront as an important route of tumor metastasis [3, 6], the major cause for treatment failure and decreased survival in cancer patients. Thus, a concerted effort is being made to identify new genetic and/or pharmacological targets for the management and treatment of lymphedema and for inhibiting the metastatic spread of cancer cells. Ideally, these treatments would improve lymphatic permeability, induce the controlled proliferation of the existing lymphatic vasculature or inhibit tumor lymphangiogenesis. The elucidation of a previously unrecognized role for AM signaling in orchestrating embryonic lymphangiogenesis considerably expands our understanding of the relatively small repertoire of molecular regulators controlling this process. Moreover, the conserved phenotypic series of mice is the first genetic, in vivo data confirming that *Ramp2* interaction with *Calcrl* is required for AM signaling during embryonic development. Therefore, further characterization of AM signaling and its pharmacological modulation via RAMP2-CLR effectors might lead to novel therapeutic strategies for the treatment of lymphedema or inhibition of tumor metastasis.

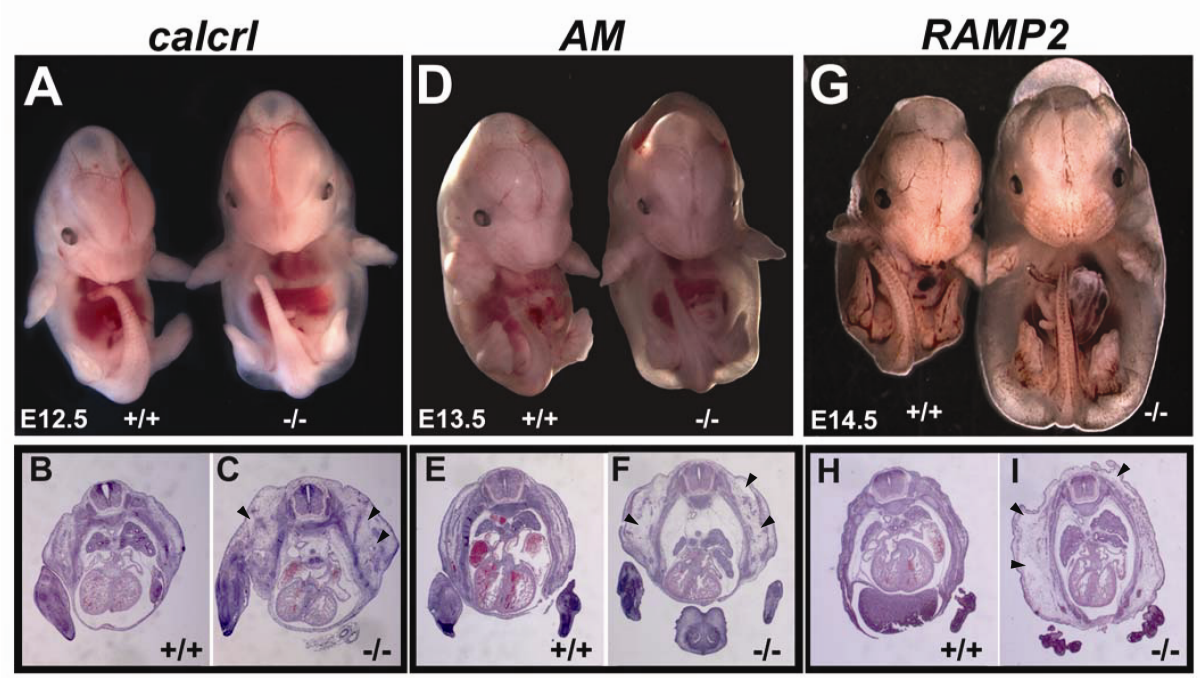

Figure 3.1: Phenotypic series of AM signaling null mice. Genetic deletion of genes responsible for AM signaling results in a phenotypic series of null mice with generalized edema and lethality at mid-gestation. Generalized interstitial edema, without hemorrhage, is observed throughout *Calcrl*$^{-/-}$ embryos at E12.5 (**A**). Similar edema is present by E13.5 in *AM*$^{-/-}$ embryos (**D**) and by E14.5 in *Ramp2*$^{-/-}$ embryos (**G**). Edema was not observed one day before onset and no viable embryos were ever recovered 1-2 days after edema onset. H&E stained transverse sections of wildtype littermates (**B, E, H**) and *Calcrl*$^{-/-}$, *AM*$^{-/-}$, and *Ramp2*$^{-/-}$ (**C, F, I**) embryos at E12.5, E13.5 and E14.5, respectively. Arrowheads indicate interstitial fluid accumulation.

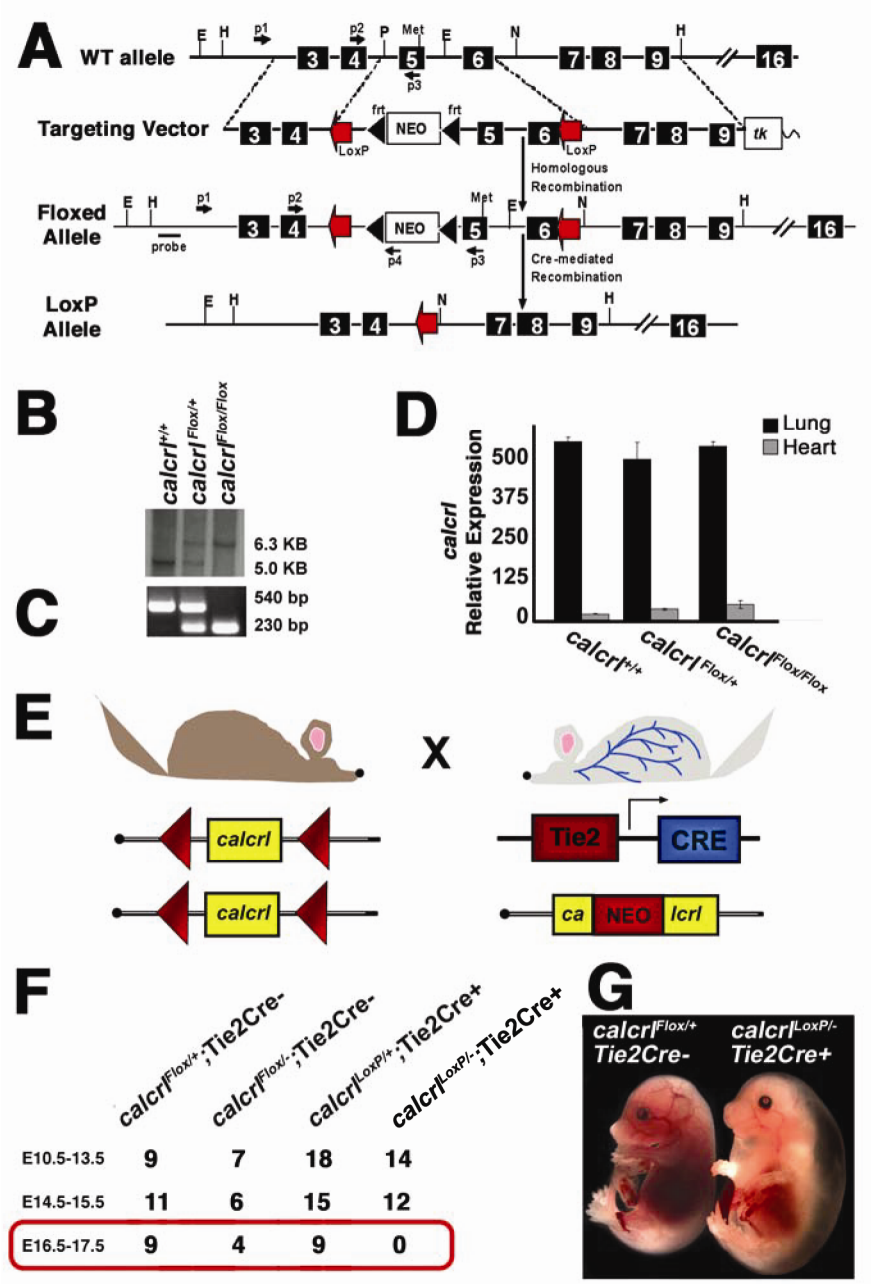
A
WT allele
Targeting Vector
Floxed Allele
LoxP Allele
NEO
tk
LoxP
Homologous Recombination
Cre-mediated Recombination
probe
B
6.3 KB
5.0 KB
C
540 bp
230 bp
D
Lung
Heart
calcrl Relative Expression
500
375
250
125
0
E
X
calcrl
Tie2
CRE
ca
NEO
lcrl
F
E10.5-13.5 9 7 18 14
E14.5-15.5 11 6 15 12
E16.5-17.5 9 4 9 0
G
Tie2Cre-
Tie2Cre+

Figure 3.2: Generation and characterization of conditional *Calcrl* line. (**A**) Schematic diagram depicting strategy used for generation of a "Floxed" *Calcrl* allele by gene targeting. The top figure shows the endogenous wildtype *Calcrl* allele. The targeting vector was designed so that LoxP sites would flank the same exons that were deleted in the Calcrl global knockout [35]. The third line depicts the targeted $Calcrl^{Flox}$ allele and the fourth line depicts the $Calcrl^{LoxP}$ allele after Cre-mediated excision. Primers used for isolation of correctly targeted ES cells and for routine genotyping are shown with small arrows. (**B**) Correctly targeted ES cells were confirmed by Southern blot analysis using the probe depicted in Fig. 2A. (**C**) PCR genotyping for the $Calcrl^{Flox}$ allele. (**D**) Quantitative RT-PCR was performed on RNA isolated from lungs and hearts of wildtype and homozygous $Calcrl^{Flox/Flox}$ mice and revealed no significant differences in the expression of the $Calcrl^{LoxP}$ allele before Cre-mediated excision. (**E**) Schematic representation of breeding scheme used to generate mice with *Calcrl* expression deleted specifically in endothelial cells by use of the Tie2Cre transgene. (**F**) Results of cross demonstrate that no viable $Calcrl^{LoxP/-}$*;Tie2Cre*+ mice were found beyond E16.5. (**G**) Compared to $Calcrl^{LoxP/+}$*;Tie2Cre*+ control littermates, the $Calcrl^{LoxP/-}$*;Tie2Cre*+ mice displayed remarkable hydrops without hemorrhage which phenocopied the global Calcrl knockout phenotype, yet often occurred substantially later at E16.5.

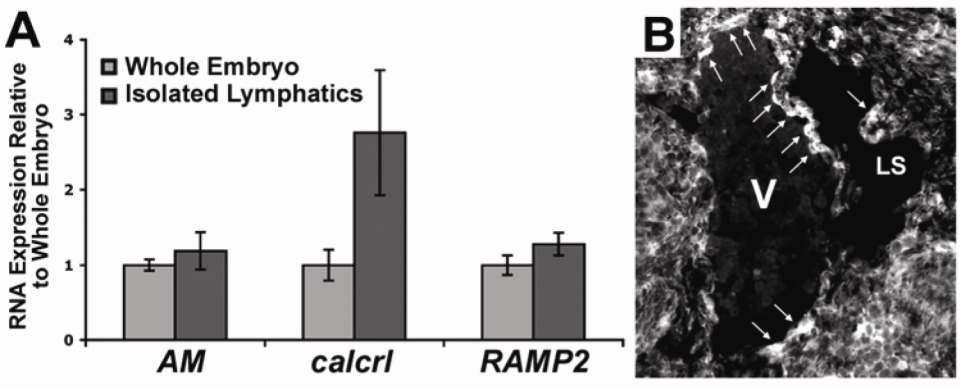

Figure 3.3: AM signaling components are expressed in adult and developing lymphatic vessels. (**A**) Isolated adult lymphatic vessels express *AM, Calcrl* and *Ramp2* at levels similar to whole embryo RNA extracts. Adult mouse lymphatic vessels were identified by the uptake of Evan's blue dye injected into the hind paw and were subsequently microdissected for RNA isolation. Expression of *AM, Calcrl* and *Ramp2* mRNAs in these vessels was comparable to mRNA levels in E10-E12.5 embryos by quantitative RT-PCR. n>4 samples, analyzed twice in triplicate. (**B**) To determine whether lymphatic endothelial cells (LECs) express AM during development, we made use of an enhanced green fluorescent protein that serves as a biological marker of *AM* gene expression in *AM* gene targeted mice[36]. Unstained transverse sections through the jugular region of an E12.5 $AM^{+/-}$ embryo show that AM is expressed in endothelial cells of both the jugular vein (V) and jugular lymph sac (LS) (arrows). Note the polarized expression of AM in the progenitor cells which will eventually migrate to form the lymph sac.

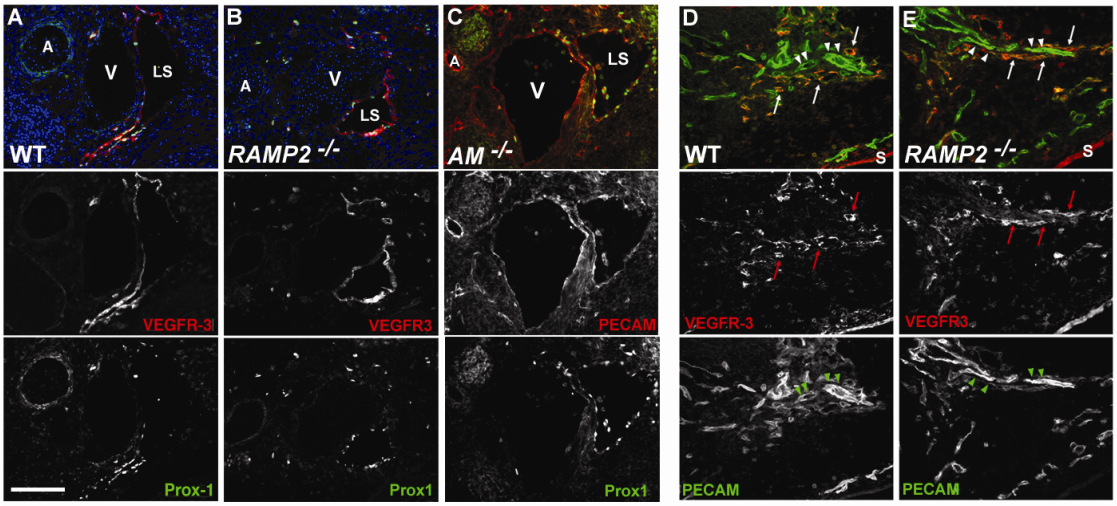

Figure 3.4: Differentiated lymph sacs and dermal lymphatics are present in AM signaling null embryos. Immunofluorescent staining of transverse sections through the jugular region (**A-C**) and sagittal sections through the skin (**D-E**) of E13.5 embryos. (**A**) Endothelial cells of lymph sacs (LS) from wildtype (WT) mice at E13.5 express VEGFR3 (red) and Prox1 (green). (**B**) Similar staining is observed in the lymph sacs of *Ramp2*$^{-/-}$ littermates. Nuclei stained with Hoechst are indicated in blue (A,B). (**C**) Prox1 (green) is also correctly co-expressed with PECAM (red) in lymph sacs of *AM*$^{-/-}$ embryos. (**D-E**) Dermal lymphatics (arrows) are present in WT (**D**) and *Ramp2*$^{-/-}$ (**E**) littermates at E14.5 as shown by VEGFR3 staining (red) and PECAM (green). Dermal blood endothelium (arrowheads) expresses PECAM, but not VEGFR3. n>6 embryos per genotype. (Carotid artery, A; Jugular vein, V; lymph sac, LS; Skin, S)

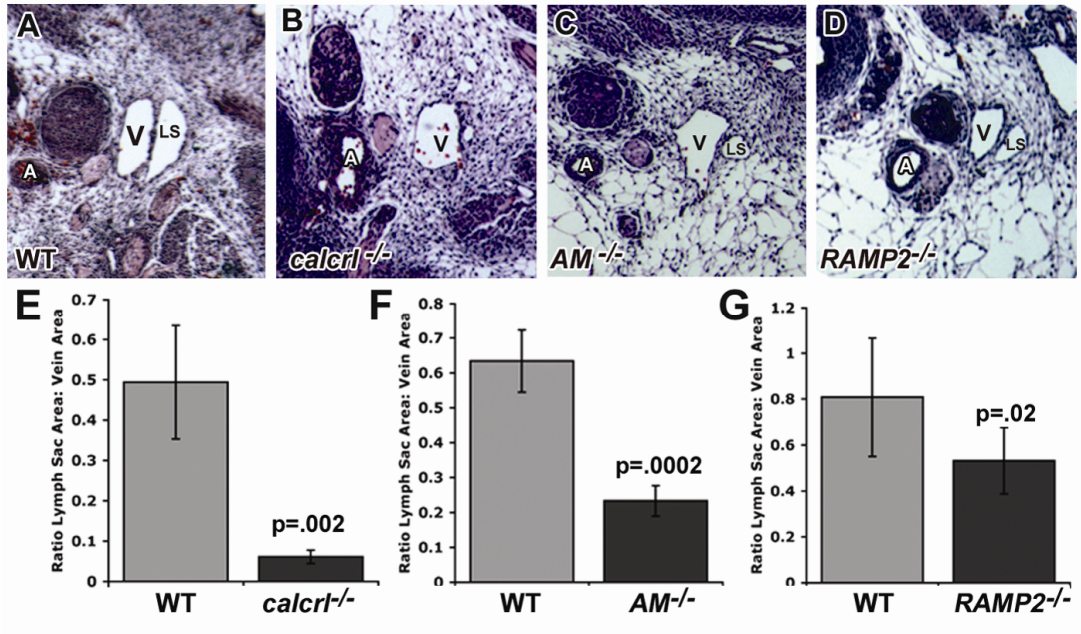

Figure 3.5: Loss of AM signaling results in hypoplastic lymph sacs. Lymph sacs of *Calcrl*$^{-/-}$, *AM*$^{-/-}$, and *Ramp2*$^{-/-}$ embryos are significantly smaller than those of their WT littermates at E13.5. (**A-D**) H&E stained sections through trasverse sections of the jugular lymph sacs (LS) of WT (**A**), *Calcrl*$^{-/-}$ (**B**), *AM*$^{-/-}$ (**C**), and *Ramp2*$^{-/-}$ (**D**) embryos at E13.5. Note the remarkable decrease in size of lymph sacs relative to jugular vein (V) and carotid artery (A) in the AM signaling mutant embryos, despite massive interstitial edema. (**E-G**) Quantitation of lymph sac area, normalized to jugular vein area in *Calcrl*$^{-/-}$(E), *AM*$^{-/-}$(**F**), and *Ramp2*$^{-/-}$ (**G**) embryos (black bars) and their WT littermates (grey bars) at E13.5. Panels E, F and G have different scales. n>6 embryos per genotype. Carotid artery, A; Jugular vein, V; lymph sac, LS. Error bars indicate SEM. p-values are from student's t-test.

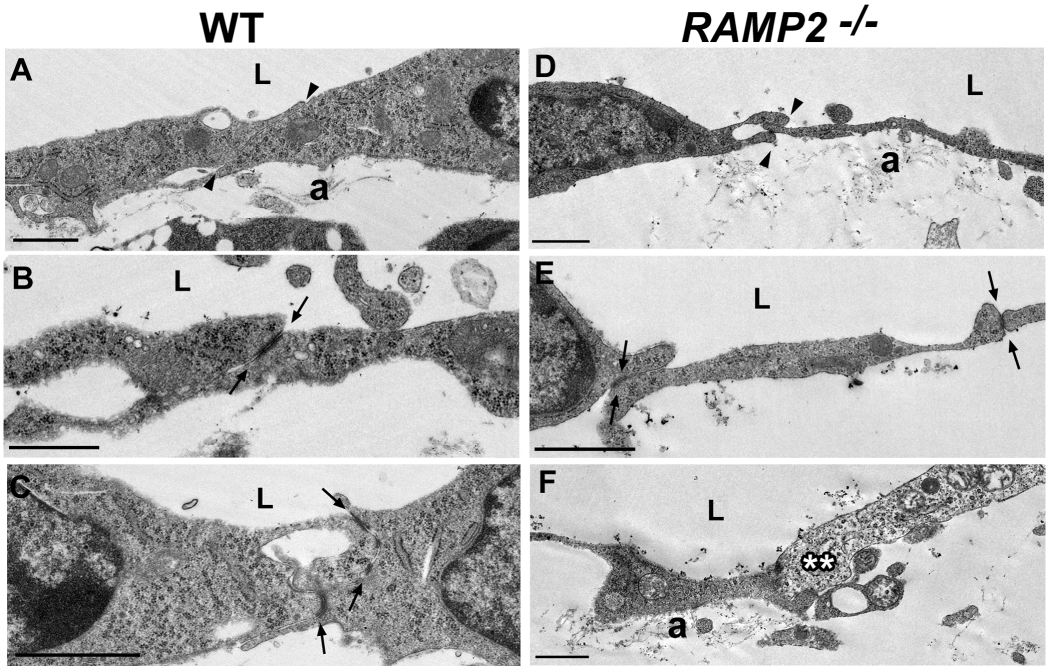

Figure 3.6: Lymphatic endothelial cells of *Ramp2*$^{-/-}$ mice are thin with otherwise normal ultrastructural features. Transmission electron microscopy of WT (**A-C**) and *Ramp2*$^{-/-}$ (**D-F**) lymph sacs at E14.5. In general, the LECs of *Ramp2*$^{-/-}$ mice appeared significantly thinner than those of wildtype littermates. Although obvious edema was present in the interstitial space surrounding both the veins and lymph sacs in *Ramp2*$^{-/-}$embryos, endothelial cells could be followed continuously around both vessel types, without obvious breaks or gaps. Other typical ultrastructual features of lymphatic vessels were identified in both wildtype and *Ramp2*$^{-/-}$ mice including: overlapping contacts (arrowheads), tight junctions (arrows) and anchoring filaments (a). Occasionally necrotic endothelial cells (**) were observed in *Ramp2*$^{-/-}$ mice, but the associated tight junctions remained structurally intact. n=2 wildype and 3 *Ramp2*$^{-/-}$. L; lumen. Bar equals 1µm.

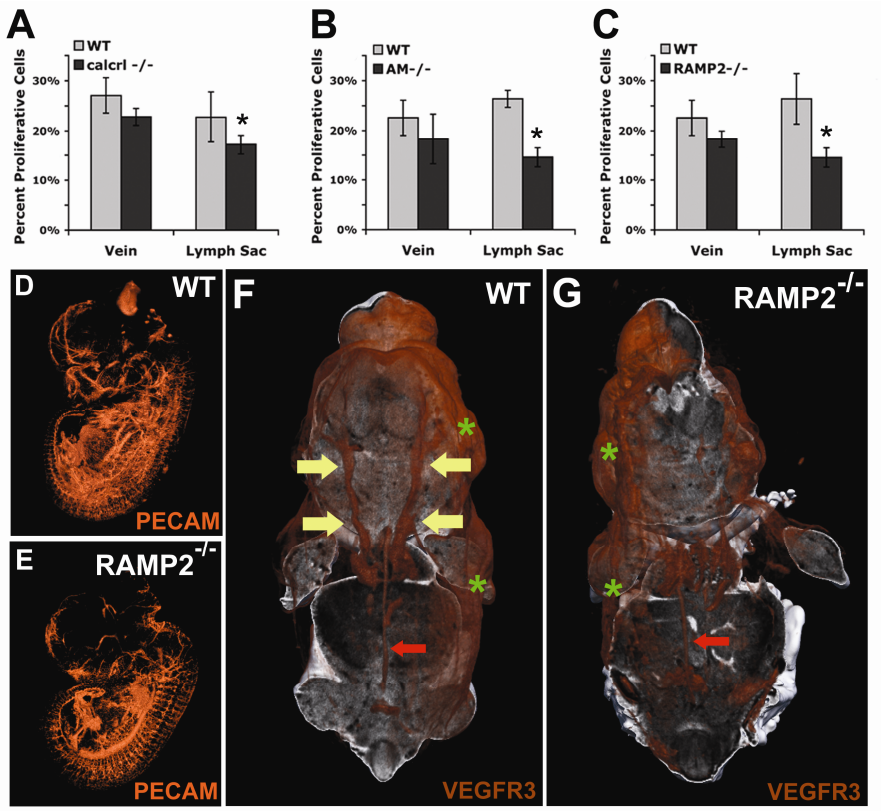

Figure 3.7: AM signaling is required for the proliferation and growth of jugular lymphatic vessels during embryonic development. (**A-C**) The percentage of proliferating cells to total cells was determined in the jugular vein and neighboring jugular lymph sac of WT (grey bars) and *Calcrl*$^{-/-}$, *AM*$^{-/-}$, and *Ramp2*$^{-/-}$ (black bars, **A-C** respectively) littermate embryos. Percent proliferative cells was defined as the number of BrdU positive endothelial cells divided by total number of endothelial cells in each vessel. Error bars indicate SEM. *, p values were generated by student's t-test and were p = 0.01 for *Calcrl*$^{-/-}$ (**A**), p = 0.004 for *AM*$^{-/-}$ (**B**), and p = 0.01 for *Ramp2*$^{-/-}$ (**C**). n>6 animals per genotype. (D-E) Whole mount immunofluorescence of developing vasculature identified by PECAM staining and visualized by 3-D optical projection tomography of wildtype (**D**) and *Ramp2*$^{-/-}$ (**E**) littermate embryos at E13.5. (**F-G**) Whole mount immunofluorescence of developing lymphatic vasculature identified by VEGFR3 staining and visualized by 3-D optical projection tomography of wildtype (**F**) and *Ramp2*$^{-/-}$ (**G**) littermate embryos at E14.5. Compare the presence of well-formed jugular lymph sacs in the wildtype embryo (yellow arrows in **F**) to the relative lack of jugular lymphatic vessels in the *Ramp2*$^{-/-}$ littermate (**G**). Note that the retroperitoneal lymph vessel (red arrows) and dermal lymphatic vessels (green asterisk) of *Ramp2*$^{-/-}$ mice appear normal compared to those of wildtype embryos. 3 embryos from 2 different litters were stained for PECAM and VEGFR3. The **Appendix** contains online links to movies for enhanced, high resolution, 3-dimensional viewing (**Appendix: Supplemental Movies 4 & 5)**.

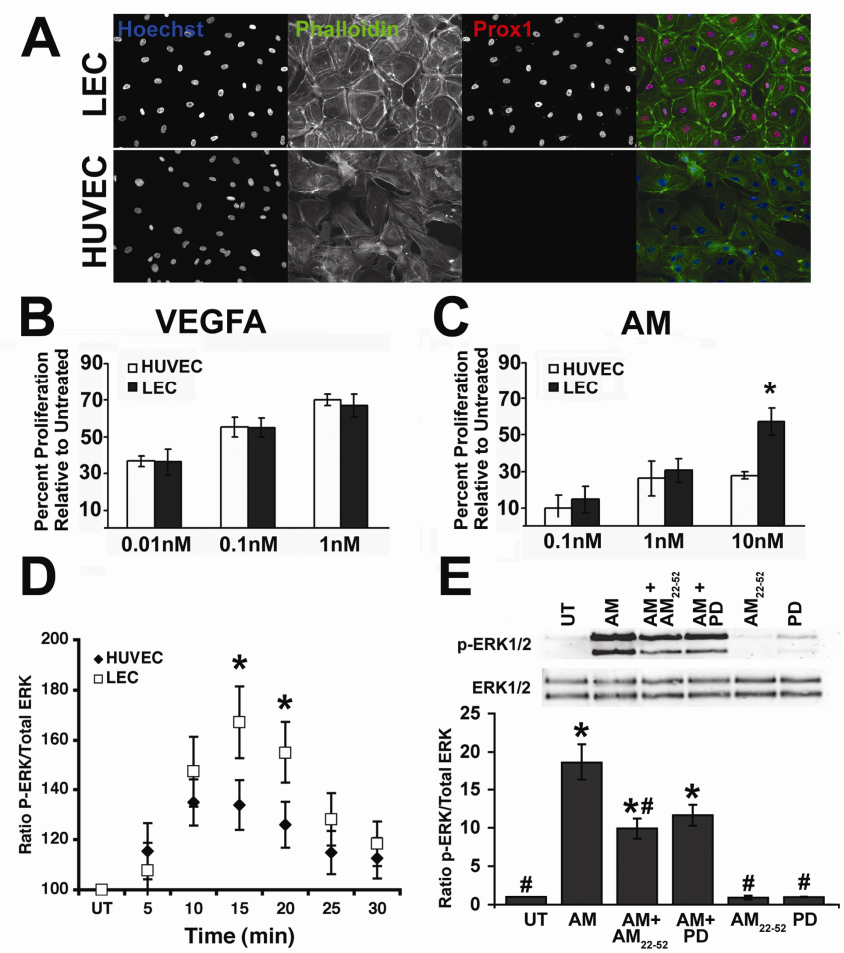

Figure 3.8: AM signaling preferentially mediates enhanced ERK activation in LECs compared to HUVECs. (**A**) Cultured LECs and HUVECs are morphologically and genetically distinct cell lines based on histology and expression pattern of Prox1. (**B**) Stimulation of HUVECs and LECs with the potent growth factor VEFGA resulted in a dose-dependant increase in cell proliferation that was not significantly different between the two cell lines. Data represent averages of 4 independent experiments, each performed in duplicate. (**C**) Stimulation of HUVECs and LECs with AM peptide resulted in a dose-dependant increase in cell proliferation that was significantly greater in LECs compared to HUVECs. * $p<0.05$ Data represent averages of 4 independent experiments, each performed in duplicate. (**D**) Stimulation of HUVECs and LECs with 10nM AM peptide resulted in a significantly greater induction of ERK phosphorylation in LECs compared to HUVECs over a 30 minute time course. * $p<0.03$ at 15 and 20 minute time points. Data represent averages of 3 independent experiments, each performed in duplicate. (**E**) Induction of ERK activation by AM stimulation in LECs was significantly reduced by the RAMP2-specific peptide inhibitor, AM22-52, and completely blocked by the MAPK inhibitor PD98057. *$p<0.05$ compared to untreated; **$p<0.05$ compared to AM treated. Data represent averages of 3 independent experiments, each performed in duplicate.

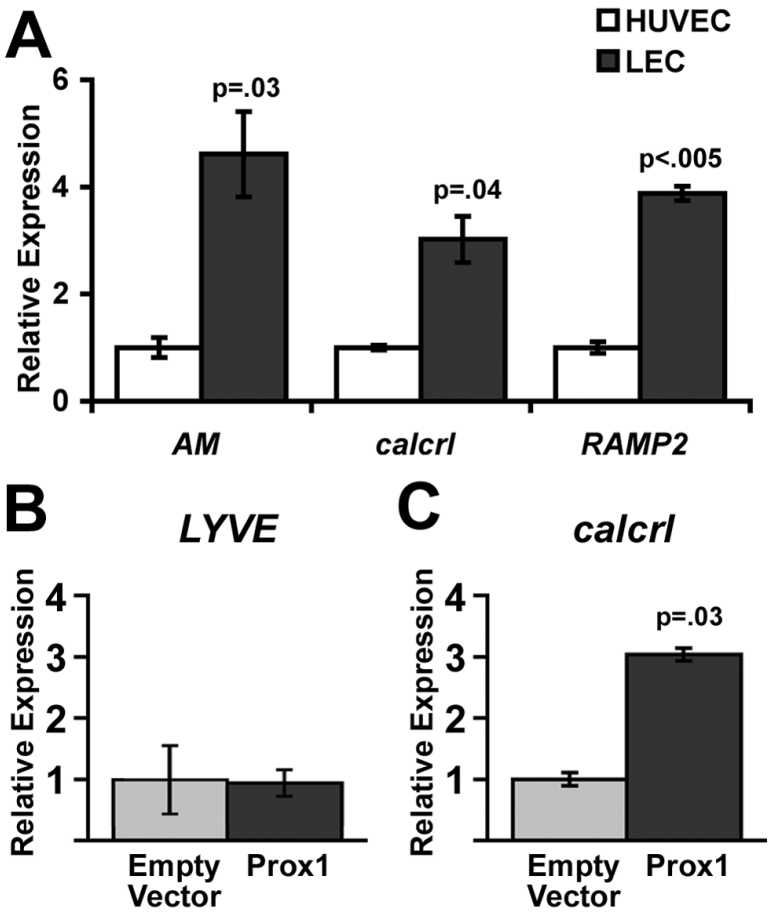

Figure 3.9: AM signaling genes are preferentially up-regulated in LECs via Prox1 induction. (**A**) Comparative gene expression analysis for *hAM*, *hCALCRL* and *hRAMP2* in cultured HUVECs versus LECs, demonstrating significantly higher levels of gene expression in LECs compared to HUVECs. Data represent averages of 3 independent experiments, each performed in duplicate. (**B**) As expected, the endogenous expression of the lymphatic-specific marker *LYVE* was not induced by transient overexpression of hProx1 in LECs. (**C**) In contrast, expression of endogenous *Calcrl* was potently induced by transient overexpression of hProx1 in LECs. Data presented in C and D are from one experiment and representative of 3 independent transfections each performed in triplicate.

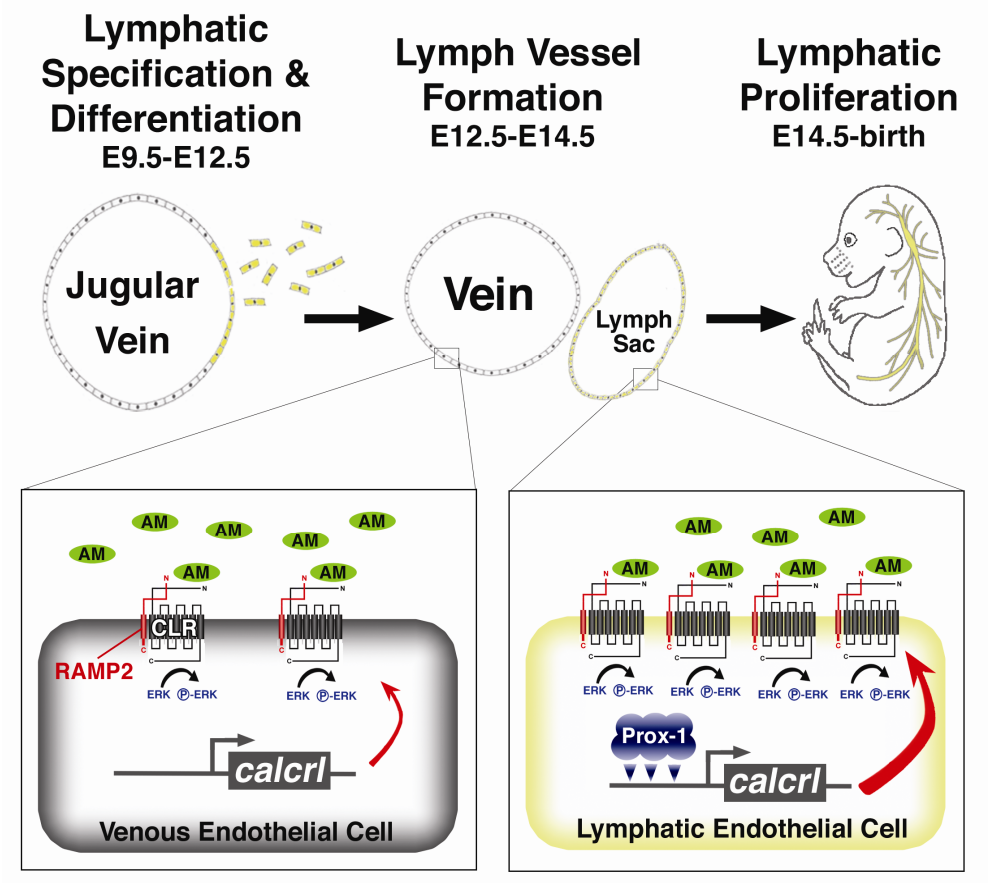

Figure 3.10: Model for role of AM signaling in lymphangiogenesis. Preferential up-regulation of AM signaling genes in lymphatic endothelial cells leads to robust AM signaling and enhanced activation of ERK phosphorylation in lymphatic versus blood endothelial cells which is essential for maintaining normal lymphatic vessel proliferation from E14.5 to birth.

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CHAPTER 4

ADRENOMEDULLIN STABILIZES THE LYMPHATIC ENDOTHELIAL BARRIER IN VITRO AND IN VIVO

Abstract

The lymphatic vascular system functions to maintain fluid homeostasis by removing fluid from the interstitial space and returning it to venous circulation. This process is dependent upon the maintenance and modulation of a semi-permeable barrier between lymphatic endothelial cells of the lymphatic capillaries. However, our understanding of the lymphatic endothelial barrier and the molecular mechanisms that govern its function remains limited. Adrenomedullin (AM) is a 52 amino acid secreted peptide which has a wide range of effects on cardiovascular physiology and is required for the normal development of the lymphatic vascular system. Here, we report that AM can also modulate lymphatic permeability in cultured dermal microlymphatic endothelial cells (HMVEC-dLy). AM stimulation caused a reorganization of the tight junction protein ZO-1 and the adherens protein VE-cadherin at the plasma membrane, effectively tightening the endothelial barrier. Stabilization of the lymphatic endothelial barrier by AM occurred independently of changes in junctional protein gene expression and *AM*$^{-/-}$ endothelial cells showed no differences in the gene expression of junctional proteins compared to wildtype endothelial cells. Nevertheless, local administration of AM in the mouse tail decreased the rate of lymph uptake from the interstitial space into the lymphatic capillaries. Together, these data reveal a previously unrecognized role for AM in controlling lymphatic endothelial permeability and lymphatic flow through reorganization of junctional proteins.

Introduction

The lymphatic vascular system is a blind-ended network of endothelial cell lined vessels that functions to maintain fluid homeostasis by unidirectionally transporting tissue fluid, extravasated plasma proteins, lipids, and cells from the interstitium to the circulatory system by way of the thoracic duct. When the lymphatic vascular system fails to function properly patients are at risk of developing serious and debilitating lymphedema. Pressure sensing, fibrillin-rich anchoring filaments that tether lymphatic endothelial cells (LECs) to the extracellular matrix contract in response to increases in interstitial pressure, thereby stretching LECs apart and facilitating lymph uptake into lymphatic capillaries. However, recent evidence suggests that LECs are also active participants in lymph transport through formation of an endothelial barrier that can regulate both ion and protein transport [1-3]. While these studies have implicated vascular endothelial growth factors A and C (VEGFA, VEGFC) and the intracellular signaling molecule cAMP as potential players in LEC permeability regulation, there remains a need to identify pharmacologically tractable targets that can efficiently modulate lymphatic permeability.

Adrenomedullin (AM) is a highly conserved 52 amino acid peptide vasodilator that is upregulated in a variety of cardiovascular conditions [4-6]. First recognized for its ability to maintain vascular smooth muscle tone, AM is also an important regulator of endothelial cell biology. For example, numerous in vitro studies have shown that AM is a potent angiogenic factor [7-9]. Our own recent studies using knockout mice have revealed that AM and its receptors are required for normal lymphatic vascular development [10].

With regard to endothelial permeability, AM treatment of human umbilical vein endothelial cell (HUVEC) monolayers dose dependently reduced hyperpermeability caused by inflammatory mediators including H_2O_2, thrombin, *E. coli* hemolysin and *S. aureus* α-toxin [11-13] The protective effect of AM on the endothelial barrier has also been shown in vivo in rat ileum exposed to *S. aureus* α-toxin and ex vivo in rabbit lungs exposed to H_2O_2 [11, 13]. Further, in the blood-brain barrier, several studies have shown that AM treatment increased transendothelial electrical resistance thereby reducing endothelial permeability [14-16]. Taken together, these data suggest AM functions as a potent factor in maintaining the blood endothelial barrier; however, whether this function is conserved in LECs and to what extent remains unknown.

The purpose of this study was to explore the role of AM in the regulation of lymphatic permeability. We evaluated the effects of AM on the expression and localization of LEC junction components. Specifically, the response to AM treatment of the tight junction molecules Zonulus Occludin (ZO-1), Claudin-5, Claudin-12, and Junction Adhesion Molecular C (JAMC) as well as the endothelial-specific adherens protein VE-cadherin were assessed. Further, the ability of AM to modulate LEC permeability was measured utilizing both in vitro and in vivo approaches. The in vivo studies exploited the technique of fluorescent tail microlymphography and provided functional validation of our in vitro observations. Together, these data establish a previously unrecognized role of AM in stabilizing of the endothelial barrier and modulating lymphatic flow in vivo.

Experimental Methods

Cell Culture

Cryopreserved adult human dermal lymphatic microvascular endothelial cells (HMVEC-dLys) were obtained from Cambrex (Walkersville, MD) and maintained in Clonetics (East Rutherford, NJ) microvascular endothelial growth medium (EGM-2MV) and endothelial basal medium (EBM), respectively. Human AM and human AM $_{22-52}$ were purchased from American Peptide (Sunnyvale, CA), Forskolin from Sigma-Aldrich, and recombinant human VEGFA from Pierce Biotechnology. Goat anti-VE-cadherin (C-19) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-ZO-1 antibody (33-9100) was obtained from Zymed (San Francisco, CA). Cy2 and Cy3-labeled secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

Immunofluorescence labeling and microscopy

For ZO-1 and VE-cadherin localization, cells were grown on 0.2% gelatin coated coverslips until they achieved confluence. The medium was changed to serum-free conditions and the cells were stimulated with AM (100 nM), VEGFA (10 ng/mL) or vehicle (0.1% BSA) for 30 min. The cells were then washed twice with PBS with calcium and then fixed/permeabilized in ice-cold ethanol for 30 min. Cells were blocked with 3% BSA in PBS for 1 h at RT and incubated with primary antibody in 3% BSA overnight at 4℃ and with appropriate seco ndary antibody for 1 h at RT. Nuclei were labeled with Hoechst 33258 (Sigma). The coverslips were mounted on glass slides and images were obtained with a Nikon E800 microscope with a

Hammamatsu ORCA-ER CCD camera using Metamorph Software (Molecular Devices) and processed in Adobe Photoshop 8.0 (Adobe).

Endothelial cell permeability

HMVEC-dLys permeability was studied as previously described [17-19]. Briefly, cells were plated at a density of 1 x 10^5 cells/cm^2 on gelatin-coated membranes (Corning Costar Transwells, 0.4 μm pore size, 6.5 mm diameter) and medium was changed every 24 h. Permeability of monolayers was measured in terms of Trypan Blue-BSA (TB-BSA) transfer [17]. At 96 h post seeding, membranes were incubated with test ligands in HBSS containing 0.03 M HEPES (HBSS/HEPES) in both the apical and basal-lateral chambers. In the apical chamber, 4% TB-BSA was added with or without test ligands followed by gentle shaking at 37℃/5% CO_2 under sterile conditions for 90 min. At the end of the incubation, samples were taken from the lower chamber and the absorbance at 590nm was measured. The relative permeability was calculated by dividing the OD of treated samples by vehicle control.

Gene Expression Analysis

Total RNA was isolated from cells using the Qiagen RNAEasy Mini Kit and DNase Treated with Promega DNase per manufactures instructions. RNA was reverse transcribed using MMV reverse transcriptase (Invitrogen). cDNA was used for semi-quantitative RT-PCR or quantitative RT-PCR using gene-specific primers, SYBR Green (Stratagene), and the Stratagene MXP3000 and MxPro Software

(Stratagene). The following murine primers sequences were used: VE-Cadherin: Forward: 5'-GGTGGCCAAAGACCCTGAC-3' Reverse: 5'-ACTGGTCTTGCGGATGGAGT-3', JAMC: Forward: 5'-GCTGGGAGAGCACATGCAA-3' Reverse: 5'-CAGGAGCTCTGGGCTCACA-3'. Primers sequences for ZO-1, Claudin-5 and Claudin-12 were designed by the report by Holmes *et al* [20]. The following human primers sequences were used: VE-Cadherin: Forward: 5'-GCCAGGTATGAGATCGTGGT-3' Reverse: 5'-GTGTCTTCAGGCACGACAAA-3. Primer sets for human ZO-1, Claudin-5, Claudin-12, and JAMC were purchased from Qiagen.

Isolation of *AM*$^{-/-}$ Endothelial Cells

The generation and characterization of mice with targeted deletion of the *Adm* gene has been previously described [21]. To isolate *AM*$^{-/-}$ endothelial cells, timed matings were established between *AM*$^{+/-}$ mice and embryos were harvested at embryonic day 13.5. Genotyping was performed as previously described [21] on genomic DNA isolated from yolk sacs. Whole embryos were treated with enzyme solution containing 2.4U/mL Neutral Protease and 150U/mL Collagenase Type II (Worthington Biochemical) at 37˚C for 30 minutes to generate a single cell suspension. Cells were incubated with 3 µg Rat anti-Mouse CD31 (BD Pharmigen) at 4˚C for 30 minutes and then washed 3 times. The cells were then incubated with Sheep anti-Rat IgG Dynabeads (Invitrogen) at 4˚C for 20 minutes and then washed 4 times. Bead-bound cells were collected for RNA purification and levels of gene expression for junctional proteins were determined as described above.

Quantitative Tail Microlymphography

The flow velocity of the dermal lymphatic vessels in the mouse tail was measured as previously described [22]. Briefly, adult male 129S6/SvEv mice were anesthetized by subcutaneous injection of avertin at a concentration of 0.5mg/gm body weight. Mice placed on a heating pad in the working space underneath a Leica MZ 16 FA dissecting microscope. The tail was immobilized on the pad with two-sided tape. Next, 1 µL of a 25% FITC-Dextran (2,000,000 kDa) with vehicle or AM peptide [10 ng/µL] was loaded into a microsyringe (Hamilton, Reno, NV) fitted with a 30-gauge needle and was injected intradermally at the tip of the mouse tail. The flow of FITC-Dextran through the dermal lymphatics was acquired with a camera fitted onto the Leica microscope every minute for 15 minutes. Flow rates were determined using offline image analysis (Adobe Photoshop 8.0 and Image J) and application of a stepwise polynomial equation [22]. The data for each subregion were fit to this equation with a least squares nonlinear regression algorithm. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Statistics

Results are represented as ± S.E.M. Statistical analyses were performed with a Student *t* test with unequal variance. A P value of $<.05$ was considered statistically significant.

Results

AM prevents VEGFA-mediated increase in lymphatic endothelial cell permeability.

HMVEC-dLys were grown to a monolayer in transwells and paracellular permeability was measured by the ability of Trypan Blue-labeled bovine serum albumin (TB-BSA) to pass through a monolayer from the apical chamber to the basal lateral chamber in the presence or absence of AM or VEGFA [19]. Treatment with AM did not change the basal permeability of the HMVEC-dLy monolayer compared to vehicle control (**Figure 4.1**). As expected, treatment with VEGFA resulted in a significant increase in permeability across the HMVEC-dLys monolayer. AM stimulation of LECs in the presence of VEGFA dose-dependently prevented the VEGFA-induced increase in permeability of the HMVEC-dLys monolayer to albumin. Therefore, although AM did not affect LEC permeability under basal conditions, it was highly effective at counteracting the permeabilizing effects of VEGFA on cultured LECs.

AM stabilizes lymphatic endothelial cell-cell junctions.

HMVEC-dLys were grown to confluence and then treated with vehicle control, AM, VEGFA, or the combination of AM with VEGFA. Vehicle treated HMVEC-dLys displayed a mosaic pattern of ZO-1 and VE-cadherin staining that was characterized by both continuous and discontinuous patches along the cell-cell membranes (**Figure 4.2A and 4.2E**). Treatment of HMVEC-dLys with AM resulted in the formation of continuous tight junction strands as indicated by the tight junction

protein ZO-1 (**Figure 4.2B**). Further, AM caused a reorganization of VE-cadherin at the plasma membrane which appeared as a linearized adherence junction band compared to vehicle treated cells (**Figure 4.2F**). As expected, treatment with VEGFA dramatically disrupted of both ZO-1 and VE-cadherin localization, indicated by gaps and an intermittent, zipper-like staining pattern (**Figures 4.2C and 4.2G**). Surprisingly, co-treatment of AM with VEGFA completely abrogated the VEGFA induced disruption ZO-1 and VE-cadherin localization at the plasma membrane (**Figure 4.2D and 4.2H**). We also noticed that AM treatment appeared to only affect the localization of ZO-1 and VE-cadherin, but not the total amount of protein present at the cellular junctions. These data demonstrate that AM functions to reorganize LEC paracellular junctions by forming a tighter paracellular seal between cells and preventing their disruption by permeabilizing agents such as VEGFA.

AM stimulation does not change the expression of junction genes at the RNA level.

RNA was isolated from LECs treated with AM or with vehicle control and gene expression was measured by quantitative RT-PCR. There was no significant difference in the expression of *ZO-1*, *Claudin-5*, *Claudin-12*, *JAMC*, or *VE-cadherin* between AM treated and vehicle control (**Figure 4.3**). Therefore, the effects of AM on LEC junction composition are independent of changes in gene expression, suggesting that modulation of lymphatic function by AM is likely to occur rapidly in vivo.

Genetic depletion of AM does not change the expression of junction genes at the RNA level.

To determine whether a genetic reduction in AM could influence the expression of junction genes in vivo, we isolated endothelial cells from *AM*$^{-/-}$ mice at embryonic day 13.5. Although somewhat reduced, the gene expression levels of *ZO-1*, *Claudin-5*, *Claudin-12*, *JAMC* and *VE-cadherin* were not statistically different in *AM*$^{-/-}$ endothelial cells compared to endothelial cells isolated from wildtype littermate controls (**Figure 4.4**). Therefore, even though *AM*$^{-/-}$ mice die at mid-gestation with vascular defects, the loss of AM in endothelial cells does not affect the expression of genes important for endothelial barrier function.

AM reduces lymphatic flow in vivo.

To address whether the permeability effect of AM on LECs could result in altered lymphatic flow in vivo we used fluorescent tail microlymphography in adult male mice. Like in humans, lymphography of the mouse tail provides a sensitive and quantitative measure of dermal lymphatic capillary flow that is largely independent of the extrinsic effects of blood flow on lymphatic function. Briefly, FITC-dextran in the presence or absence of AM was injected into the interstitial space of the mouse tail, and the ability of the dextran to enter the lymphatic capillaries was monitored over time. Consistent with previously published results from other groups [22-24], the basal lymphatic flow rates in the wild-type SvEV129/6 mouse tail was 0.78 mm/min (**Figure 4.5**). When AM was co-administered with the FITC-dextran, we consistently found a significant decrease in lymphatic flow rates,

with an average uptake rate of 0.52 mm/min. Moreover, co-administration of AM with VEGFA was able to completely inhibit VEGFA-mediated increases in lymphatic permeability. Therefore, these data demonstrate that AM can potently reduce lymphatic flow rates in vivo.

Discussion

In the present study, we demonstrate that AM stabilizes the barrier function of LECs both in vitro and in vivo. AM dose-dependently prevented VEGFA-mediated increases in permeability of cultured HMVEC-dLys to albumin. Molecularly, AM treatment reorganized ZO-1 and VE-cadherin at the plasma membrane to form continuous cell-cell contacts, thereby limiting paracellular transport. In addition, we showed that exogenous AM peptide effectively slowed lymphatic flow rates in the mouse tail basally and in the presence of VEGFA. Importantly, the ability of AM to modulate lymphatic barrier functions was independent of changes in gene expression of tight junction and adherens junction components both in vitro and in a genetic knockout mouse model.

Previous studies have shown that AM can stabilize the endothelial barrier of various blood vascular beds during inflammatory conditions (H_2O_2, thrombin, *S. aureus* α-toxin, *E. coli.* Hemolysin) and thus presumably reduce edema formation [9, 11-13]. However in another study, AM increased inflammatory edema accumulation caused by substance P and bradykinin [25]. Although these studies were not able to separate the direct anti-inflammatory functions of AM from its effects on vascular permeability, our study sheds new light of the in vivo functions of AM in vascular

beds that contain both blood and lymphatic capillaries. We found that the ability of AM to maintain barrier function is not limited to blood endothelial cells but also affects LECs. Because lymphatic uptake was significantly reduced after co-administration of AM in vivo, our results suggest an essential role for AM in perpetuating inflammatory edema.

The VEGF family members VEGFA and VEGFC have both been shown to modulate lymphatic permeability. Targeted overexpression of VEGFA in the mouse epidermis exacerbated lymphatic leakage after acute UVB irradiation [3], while polymorphisms in the VEGFA promoter region leading to enhanced VEGFA expression have been attributed to hydrocele development in lymphatic filariasis [2]. In addition, VEGFC has been demonstrated to reduce the transendothelial electrical resistance (TEER) of a LEC monolayer [1]. While both VEGFA and VEGFC increase lymphatic permeability, AM represents one of the first angiogenic factors that can stabilize the lymphatic endothelial barrier. Therefore, the local interactions between VEGF family members and AM in regulating blood and lymphatic microcirculation warrant additional investigation.

We have previously shown that AM and its receptors are essential for the development and function of the lymphatic vasculature [10]. AM transduces its signal through a G-protein coupled receptor, calcitonin receptor-like receptor (CLR= protein; *Calcrl*=gene) when the receptor is associated with receptor activity modifying protein 2 (RAMP2) [26]. Mice that have targeted deletions of the genes that encode for either AM, CLR, or RAMP2 die at mid-gestation with generalized edema that is characterized by diminished proliferation of LECs [10]. A conditional

knockout of *Calcrl* in endothelial cells using the Cre-LoxP homologous recombination system also resulted in a similar phenotype, supporting an essential function for AM signaling in endothelial cells [10]. Our studies, and those of others [27, 28], have shown that AM and its receptors are intrinsically enriched in LECs compared to blood vascular endothelial cells. Therefore, the enhanced sensitivity of LECs to local AM provides an explanation, in part, for the remarkable phenotype of the null mice. Our current study extends our understanding of AM function in LECs and suggests that an additional factor leading to the embryonic edema of the null mice may be leaky, hyperpermeable lymphatic capillaries that are unable to support lymphatic flow in vivo. Consistent with findings from other groups [9, 16], the fact that isolated endothelial cells lacking AM had normal expression of genes encoding junctional proteins further demonstrates that the effects of AM on the endothelial cell barrier are largely mediated by structural reorganization of the plasma membrane rather than changes in gene expression.

The unique molecular interface formed between the association of CLR with RAMPs provides a distinctive target for pharmacological drug design. In fact, two compounds that target the CLR-RAMP1 interface are currently in clinical trials for the treatment of migraine pain associated with calcitonin gene related peptide biology [29-32]. Our current study suggests that modulation of AM activity through small molecule targeting of the CLR-RAMP2 complex could provide an effective means of altering lymphatic permeability for either the treatment of lymphedema or the inhibition of tumor metastasis through the lymphatic vasculature.

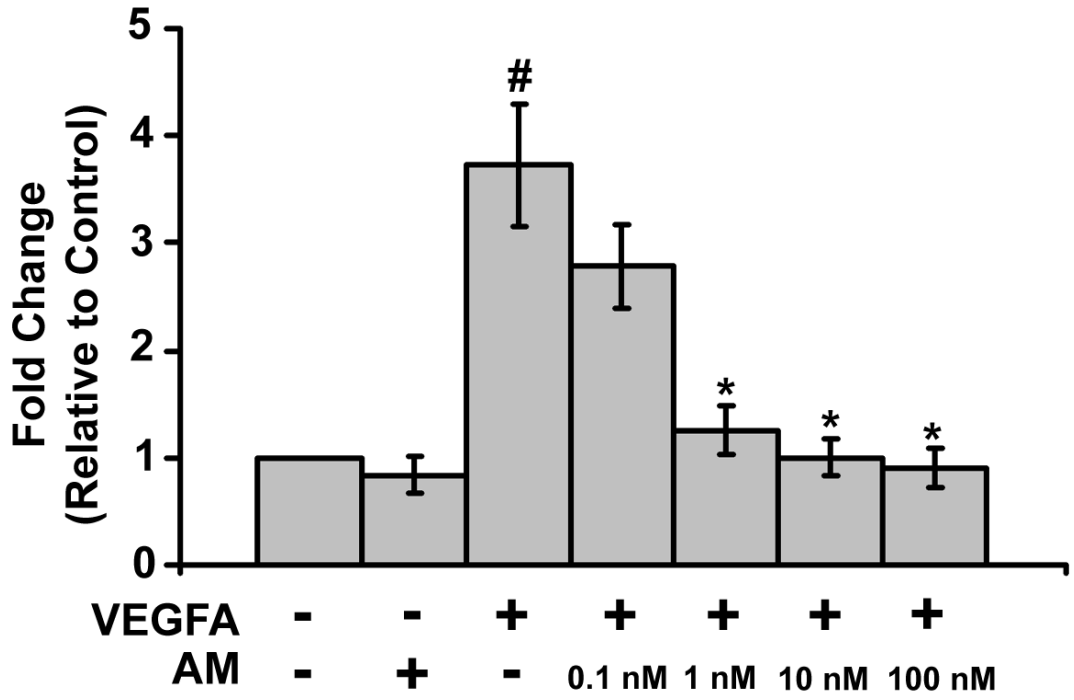

Figure 4.1: Effects of AM on the permeability of HMVEC-dLy monolayers. Cells were treated with 0.1% BSA alone, AM (100 nM), VEGFA (10 ng/mL), or VEGFA with increasing concentrations of AM (0.1 nM-100 nM) for 90 min. Relative changes in permeability were determined by measuring TB-BSA transfer for each treatment normalized to vehicle. control. #P<0.01 compared to vehicle control. *P<0.01 compared to VEGFA treated. Data represented as mean +/- SEM from 3 independent experiments, each performed in triplicate.

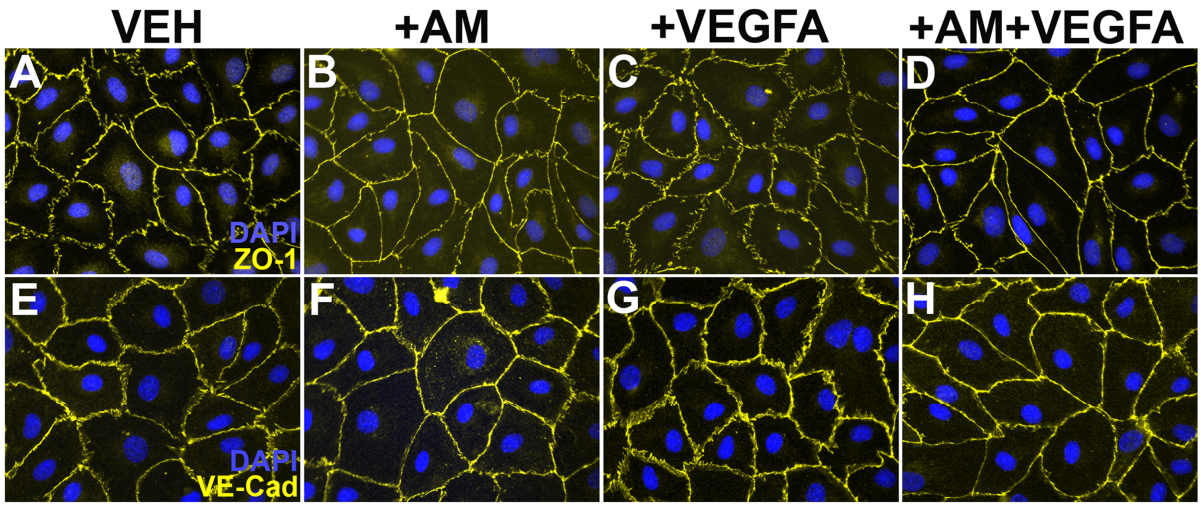

Figure 4.2: AM stabilized the endothelial barrier in HMVEC-dLys in the presence of VEGFA. Treatment with 0.1% BSA (Veh.) showed intact monolayer as visualized by ZO-1 (**A**) and VE-cadherin (**E**). Stimulation with 100 nM AM resulted in linearization of both ZO-1 (**B**) and VE-cadherin (F) at the cell-cell junction. VEGFA (10 ng/mL) treated cells displayed discontinued ZO-1 (**C**) and VE-cadherin staining (**G**). Co-treatment of AM with VEGFA stabilized ZO-1 (**D**) and VE-cadherin (**H**); completely preventing VEGFA-mediated barrier disruption. Original magnification 400X. Each image is representative of each experimental condition imaged from 4 separate fields repeated 3 times in triplicate.

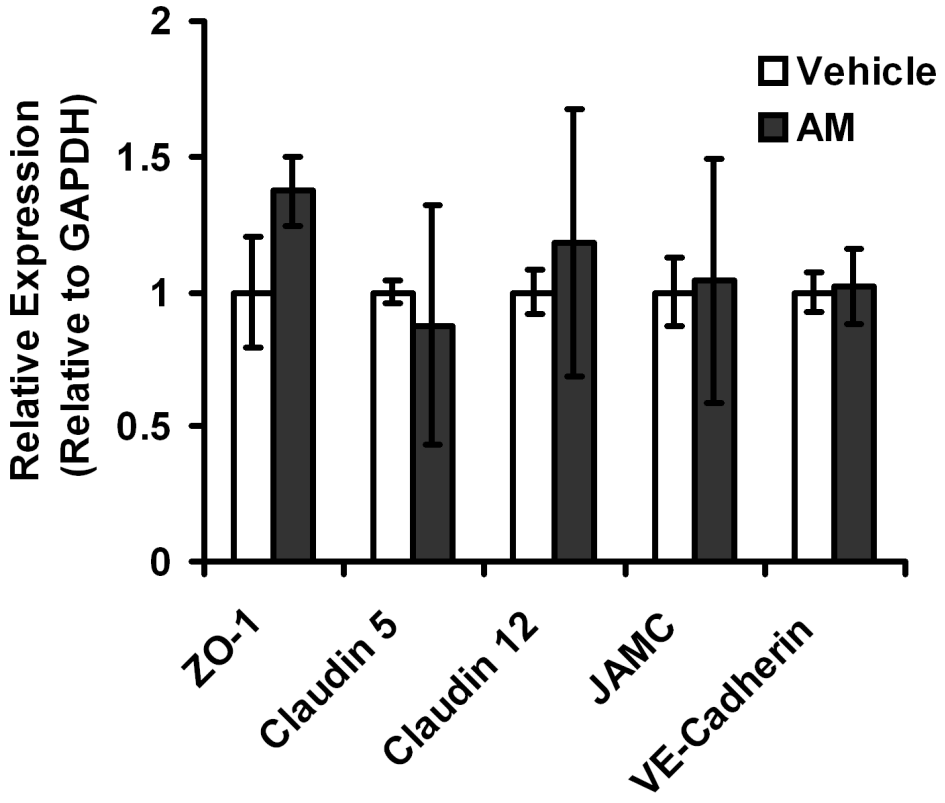

Figure 4.3: Stimulation of HMVEC-dLys with AM does not affect gene expression of junction components. Total RNA was isolated from HMVEC-dLys treated with 0.1% BSA or 100 nM AM and the gene expression of *ZO-1*, *Claudin-5*, *Claudin-12*, *JAMC*, and *VE-cadherin* were assessed relative to GAPDH. No statistical significant differences were found between the two groups suggesting the effect of AM on barrier function is independent to changes in gene expression of junction components. n>3 with each assay performed in duplicate.

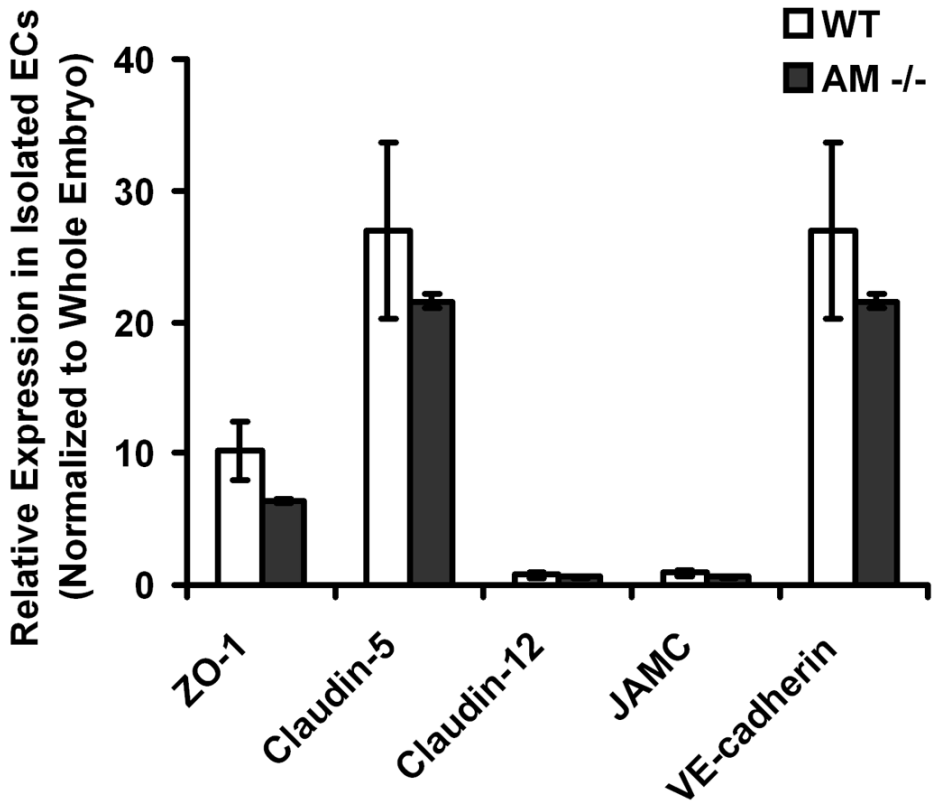

Figure 4.4: Genetic loss of AM does not affect gene expression of junction components in vivo. Using magnetic bead purification, endothelial cells were isolated from *AM*$^{-/-}$ and wildtype littermate embryos at embryonic day 13.5. The gene expression of *ZO-1*, *Claudin-5*, *Claudin-12*, *JAMC*, and *VE-cadherin* were determined and normalized to their expression in whole embryo. Although *AM*$^{-/-}$ endothelial cells had somewhat diminished gene expression levels, there were no statistically significant differences compared to wildtype endothelial cells. n>3 with each assay performed in duplicate.

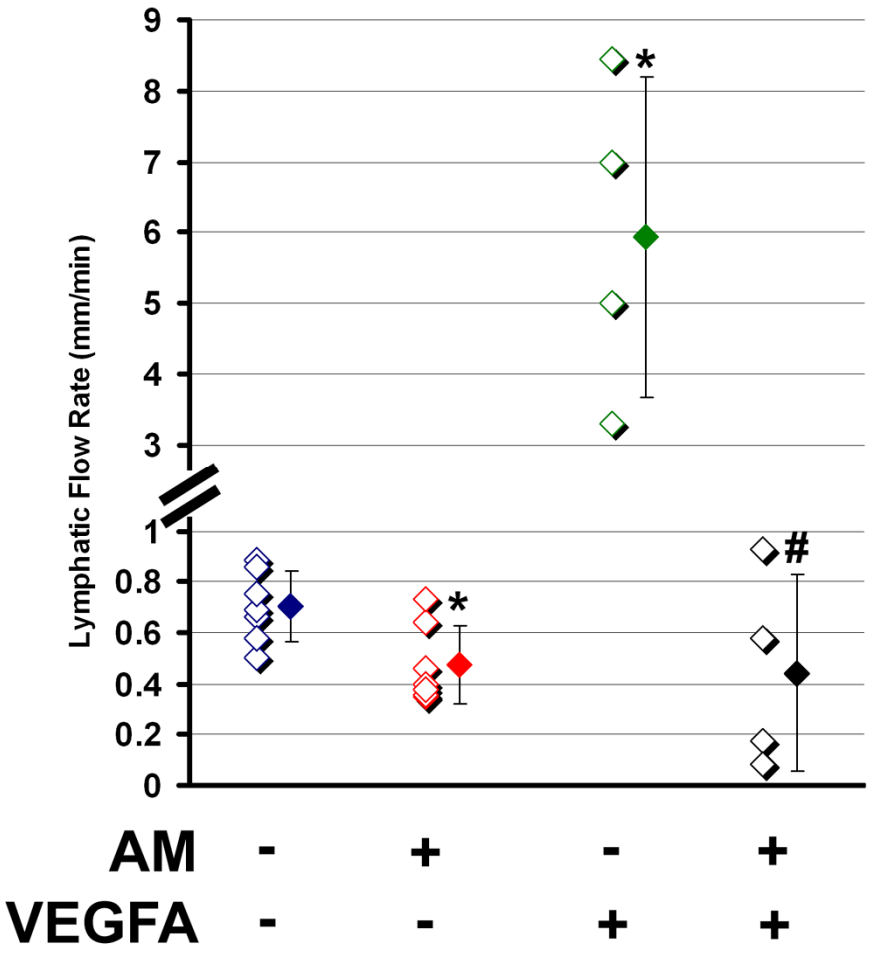

Figure 4.5: AM decreases lymph uptake into the lymphatic capillaries in the mouse tail. Tails of SvEv129/6 wildtype mice were injected with 200 kDa FITC-Dextran and movement of the labeled-dye into the lymphatic capillaries was measured over time. The rate of uptake in the absence of AM (100nm) was 0.78mm/min while the rate of uptake in the presence of AM was significantly decreased to 0.52mm/min. Co-treatment of AM with VEGFA (1.0µM) completely abrogated VEGFA mediated increase in lymphatic permeability. P*<0.01 to Veh. P<0.01 to VEGFA treated. n>4 for each treatment group.

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CHAPTER 5

VASCULAR SMOOTH MUSCLE CELL DELETION OF CALCITONIN RECEPTOR-LIKE RECEPTOR

Abstract

Adrenomedullin (AM) and calcitonin gene-related peptide (CGRP), two members of calcitonin peptide family, are secreted peptides that play important roles in the development, function, and maintenance of the cardiovascular system. During several cardiovascular stresses like hypertension, renal failure, and myocardial infarction, AM and CGRP plasma levels are robustly increased, suggesting a cardioprotective homeostatic role. Specifically, the vasodilatory activities of AM and CGRP may function to counteract the arterial vasoconstriction in hypertensive patients.

The 7-transmembrane G-protein coupled receptor calcitonin receptor-like receptor (CLR=protein name, *Calcrl*=gene name) is highly expressed in vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) and mediates the cellular effects of both AM and CGRP. We have previously shown that global deletion of *Calcrl* results in embryonic lethality, thereby precluding the ability to study the effects of *Calcrl* deletion during adult cardiovascular disease. To overcome the embryonic lethality and provide an animal model in which the role of AM and CGRP in VSMCs could be studied, VSMC-specific *Calcrl* knockout mice were generated. VSMC-specific *Calcrl* knockout mice were born at expected Mendelian ratios with no obvious developmental defects. We found no significant difference in basal blood pressure or cardiac function in VSMC-specific knockout mice compared to controls. Unexpectedly, response to phenylephrine-induced vasoconstriction was blunted in knockout mice. Together, these data demonstrate that *Calcrl* expression in VSMCs

is not required for embryonic survival or maintaining basal vascular tone and suggests that other hemodynamic mechanisms may compensate for loss of *Calcrl*.

Introduction

Calcitonin receptor-like receptor (CLR=protein name, *Calcrl*=gene name) is a receptor for two potent vasodilator peptides, adrenomedullin (AM) and calcitonin gene-related peptide (CGRP). Downstream signaling pathways for AM and/or CGRP signaling vary in both intracellular pathways and physiological effects, dependent upon the tissue type [1]. However, in most tissues, including vascular smooth muscle cells (VSMCs), the signaling cascade is most likely initially mediated by coupling of CLR to $G_s\alpha$, activation of adenylate cyclase, and subsequent production of the second messenger cAMP to orchestrate downstream effectors (rev. in [2]).

CLR is a seven-transmembrane GPCR and a member of the family of Class II GPCRs which, among others, includes calcitonin receptor (CTR), PTH 1 and 2 receptors, and VPAC1 [3, 4]. Like other Class II GPCRs, the binding specificity of CLR is dictated by the formation of a heterodimer with a family of single-pass transmembrane proteins called receptor affinity modifying proteins (RAMPs) [5]. In this non-classical signaling paradigm, the RAMP1-CLR heterodimer functions as the receptor for CGRP while the RAMP2 or RAMP3-CLR complex constitutes an AM receptor [6]. AM signaling through RAMP2-CLR is essential for survival since mice with genetic deletions of *AM*, *Ramp2*, or *Calcrl* all encounter embryonic lethality with severe cardiovascular defects [7-9]. In contrast, mice lacking CGRP signaling

through CLR by gene targeted deletion of RAMP1 survive to adulthood. [10],Li M in preparation 2009]. Together, these data suggest that signaling through CLR can lead to divergent downstream effects that are ligand and RAMP dependent.

CLR is highly expressed in the tissues of the cardiovascular system including the vasculature, lungs, and heart [11, 12]. Signaling through CLR in each of these tissues is essential for proper development of the cardiovascular system since mice with targeted deletion of *Calcrl* die during mid-gestation with thinner VSMC walls, hypoplastic lymph sacs, and disorganized hearts [8, 9]. In adulthood, activation of CLR by either AM or CGRP functions to maintain homeostasis by counteracting increases in blood pressure by causing a potent and long lasting relaxation of the VSMC layer wall [13-16]. Notably, during cardiovascular stresses that result in changes in blood pressures (including pregnancy, sepsis, congestive heart failure, hypertension, and kidney failure) plasma levels of both AM and CGRP are elevated, suggesting that signaling of these peptides through CLR plays a homeostatic role in the cardiovascular system (rev. in [2, 17]).

Studies exploring the mechanism by which CLR mediates its hypotensive responses are complex and profoundly affected by the method and dose of agonist administration as well as the tissue bed and species examined [13, 18-22]. Agonist binding to CLR relaxes vascular smooth muscle tone in two ways: i) directly through CLR activation on VSMCs leading to activation of cAMP and PKA, which in turn causes opening of K+ channels and activation of Ca2+ sequestration mechanisms or ii) indirectly through activation of CLR on ECs to initiate secretion of nitric oxide (NO), which acts upon VSMC to cause relaxation [23-25]. However, determining

which of these pathways is most important for mediating the hypotensive response remains unclear [26-28].

The purpose of this study was to examine the role of CLR signaling in vascular smooth muscle cell development and function. Specifically, we aimed to more clearly define role of CLR in vasorelaxation and hypotension. To achieve these goals, we used the Cre-LoxP system to generate VSMC-specific *Calcrl* null mice, thereby eliminating signaling for both AM and CGRP in VSMCs. All mice were born at the expected Mendelian ratios and survived well into to adulthood, demonstrating that expression of *Calcrl* in VSMCs in not required for normal embryonic development or survival. Furthermore, we found no significant differences in basal vascular tone or cardiac function between knockout and control mice. Interestingly, knockout mice displayed a decreased vasodilatory response to phenylephrine compared to control mice, suggesting that compensatory mechanisms may be in place to adjust to lack of AM and CGRP signaling in VSMCs.

Experimental Methods

Animals

The generation of *Calcrl*$^{Flox/Flox}$ mice has been previously described and were maintained on the C57B/6 genetic background [9]. C57BL/6 x SJL-Tg(Tagln-cre)1Her (*SM22-Cre*) mice were obtained from the Jackson Laboratory [29]. Vascular smooth muscle- specific *Calcrl*-null animals were generated by crossing *Calcrl*$^{Flox/Flox}$ with heterozygous *SM22Cre*$^{+}$ mice (*Calcrl*$^{+/+}$ x *SM22Cre*$^{+}$). The resulting male *Calcrl*$^{+/LoxP}$;*SM22Cre*$^{+}$ mice were bred to female *Calcrl*$^{Flox/Flox}$ mice,

generating the experimental (*Calcrl*$^{LoxP/LoxP}$;*SM22Cre*$^{+}$) and control (*Calcrl*$^{+/LoxP}$;*SM22Cre*$^{+}$) littermate mice used for this study. All mice used for this study were 3-4 month old male mice on a mixed genetic background. PCR genotyping was performed using genomic toe DNA to verify the Cre-mediated recombination of floxed *Calcrl* allele. Primer sets were used to detect the *SM22Cre* transgene, the *Calcrl* allele, and the recombined *Calcrl* floxed allele as previously described [9, 29].

All experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Gene Expression Analysis

To verify that the Cre-mediated excision event of the *Calcrl* floxed allele specifically down-regulated expression of *Calcrl* in vascular smooth muscle cells, RNA was isolated from tissues of experimental and control mice including the aorta, heart, bladder, and skeletal muscle. Because the expression of Calcrl is very high in endothelial cells (ECs), prior to RNA isolation, the advential and endothelial layers of the aorta were removed by enzymatic digestion with Type 2 collegenase (Worthington Biochemical Corporation) to obtain purified VSMC as previously described [30]. cDNA was made using Invitrogen MMLV reverse transcriptase and RT-PCR was performed with following primers and probe was used: *Calcrl* Forward: 5'-CAAGATCATGACGGCTCAATA-3'; *Calcrl* Reverse: 5'-CGTCATTCCAGCATAGCCAT-3'; *Calcrl* internal probe: 5'-FAM-

CATGCAGGACCCCATTCAACAAGCAT-TAMRA-3'. *Calcrl* gene expression was normalized to rodent GAPDH expression (Applied Biosystems) Cat#4308313).

Blood Pressure Measurements

Heart rates and blood pressures were measured on unanesthetized mice by the Hatteras Instruments computerized tail cuff system [31]. For drug studies, intra-arterial catheter measurements were taken as previously described [32]. Briefly, mice were anesthetized with 1-1.5% isoflurane and placed on a heating pad to maintain body temperature at 37℃. Catheters were inserted into the right carotid artery and the left jugular vein. Baseline blood pressure was established before each drug was administered and allowed to re-establish before the next drug was administered. Phenylephrine (Sigma) was delivered at 30, 60, or 90µg/kg in 50µl saline. Maximal change in blood pressure response was measured and in all treatments baseline blood pressure was re-established within 2 min.

Echocardiography

Transthoracic echocardiography (TTE) was performed on 3-4 month old male mice at baseline using a 30-MHz probe and Vevo 660 Ultrasonography (VisualSonices). Mice were anesthetized with 1-1.5% isoflurane. Body temperature was maintained at 37℃ by placing mice under a heating lamp and heart rate was closely monitored. The heart was imaged in the two-dimensional mode in the parasternal long-axis view. Measurements were obtained by positioning an M-mode curser perpendicular to the interventriculuar septum and the posterior wall of

the left ventricle at the level of the papillary muscle. All measurements were acquired and analyzed according to the American Society of Echocardiography guidelines [33].

Histology

Hearts were dissected with the corresponding ascending aorta and fixed in 4% paraformaldehyde and 6-10 µm sections were prepared. The sections were stained with H&E to assess gross morphology of the heart. Cell thickness of the VSMC layer of the thoracic aorta was measured. To measure heart-to-body ratios, mice were weighed and the hearts were dissected, washed with PBS, separated into the 4 chambers, and weighed.

Metabolic Assessment

Mice were contained in metabolic cages for 3 days with free access to food and water. At each 24 time point urine volume, water consumption, and mouse weight was measured.

Statistics

Results are represented as ± S.E.M. Statistical analyses were performed with a Student *t* test with unequal variance. A P value of <0.05 was considered statistically significant.

Results

Generation of VSMC-Specific *Calcrl* Deficient Mice.

To study the developmental and physiological role of CLR in vascular smooth muscle cells (VSMCs), we generated VSMC-specific *Calcrl* null mice. *Calcrl*$^{Flox/Flox}$ mice were bred to *Calcrl*$^{+/LoxP}$;*SM22Cre*$^{+}$ mice that were heterozygous for the *SM22Cre* transgene that is specifically expressed in VSMCs [29, 34]. Genotyping was performed with genomic toe DNA from pups generated from the aforementioned cross and verified the four expected genotypes (**Figure 5.1 A**). Importantly, only mice that expressed the *SM22Cre*$^{+}$ transgene generated the recombined *Calcrl* allele through the Cre-mediated excision of exons 5 and 6. For all experiments, *Calcrl*$^{LoxP/LoxP}$;*SM22Cre*$^{+}$ (VSMC-specific *Calcrl* null mice) and *Calcrl*$^{+/LoxP}$;*SM22Cre*$^{+}$ served as experimental and control mice, respectively. The *SM22Cre*$^{+}$ transgene was expressed in both control and experimental mice to ensure Cre recombinase toxicity could not contribute to differential phenotypes [35].

The resultant pups were born at expected Mendelian ratios and survived to adulthood without any obvious phenotypic defects (**Figure 5.1A**). To verify that the *SM22Cre* mediated excision of the floxed *Calcrl* allele resulted decreased *Calcrl* gene expression, rtPCR was performed with RNA isolated from various mouse tissues including the VSMC-rich thoracic aorta. As shown in **Figure 5.1B**, *Calcrl* gene expression was significantly decreased in the aortic VSMCs as well as the bladder and the heart of *Calcrl*$^{LoxP/LoxP}$;*SM22Cre*$^{+}$ mice relative to controls. Importantly, the SM22 Cre mediated excision was exclusive to these tissues as

previously reported [34]. Together, these data demonstrate that the expression of *Calcrl* in VSMCs is not required for embryonic development and survival.

Assessment of Basal Cardiovascular Function.

To determine the effect of lack of *Calcrl* on VSMC function, blood pressure and heart rate was measure with tail-cuff on conscious adult mice. A shown in **Figure 5.2A-B**, we found no significant difference between knockout and control animals. Furthermore, to assess basal cardiovascular function and morphological parameters, echocardiography was performed on anesthetized mice from both experimental and control groups. As shown in **Table 5.1A**, no significant differences were found for any of the physiological parameters measured including aortic diameter/contraction between the knockout and control mice. All of the measured parameters fell within the range of previously reported values [36, 37]. Further, no differences were found in the gross histology of the heart (**Figure 5.3**) or in the cell thickness of the ascending aorta (7.0 ± 0.1 vs 6.9 ± 0.1) between knockout and control mice. Lastly, **Table 5.2B** demonstrates no significant difference in body weight, heart size or metabolic responsiveness/kidney function between the two groups. Taken together, these data strongly support that under basal conditions there are no cardiovascular deficiencies in mice lacking *Calcrl* in VSMCs.

Responsiveness of the Vascular Smooth Muscle to Phenylephrine.

The lack of a basal cardiovascular phenotype suggested that other vasoregulatory VSMC mechanisms may have compensated for the loss of two

potent vasodilators CGRP and AM. To test VSMC responsiveness in knockout and control mice, intra-arterial blood pressure measurements were taken in anesthetized mice after challenge with the α1-adrenergic receptor agonist, phenylephrine, over increasing doses of 30ug/kg, 60ug/kg, and 90ug/kg. While both knockout and control mice showed the expected increases in MAP and corresponding decreases in HR, the percent change relative to baseline in the knockout mice was significantly less compared to control mice for both MAP and HR (**Figure 5.4A-B**). Together, these data demonstrate that the lack of *Calcrl* in VSMCs resulted in a reduced response to phenylephrine, supporting a significant, yet unexpected role for CLR in VSMCs.

Discussion

The G-protein coupled receptor CLR functions as a receptor for two multifunctional, vasodilators AM and CGRP. Many of the physiological functions of AM and CGRP occur in the cardiovascular system where CLR is expressed on the surface of ECs, VSMCs, and cardiomyocytes [11]. Global *Calcrl* knockout mice are embryonic lethality with defects in the proliferation of cardiovascular tissues including the lymphatic endothelium, vascular smooth muscle wall, and myocardium [8, 9]. To determine which cell type the loss of *Calcrl* leads to embryonic lethality, our lab has generated a conditional allele of *Calcrl*. By breeding to either EC-specific; cardiomyocyte-specific or VSMC-specific Cre lines, we are able to compare and contrast the outcomes and phenotypes in order to better elucidate the cellular role of *Calcrl* in development and cardiovascular physiology. Remarkably, mice

lacking *Calcrl* specifically in ECs, using the *Tie2Cre* transgene, completely recapitulate the phenotype of the global *Calcrl*, *AM* and *Ramp2* knockout mice strongly suggesting that AM signaling through the CLR-RAMP2 receptor complex in endothelial cells is necessary for embryonic development [9]. We have also shown that mice with *Calcrl* deleted in cardiomyocytes, using either the *αMHCCre* or *cTNTCre* transgenes, survive to adulthood (Dackor R, unpub. obs.). In this study, we further show that mice which specifically lacked *Calcrl* in VSMCs, using the *SM22Cre* transgene, survive to adulthood. Therefore, these comparative studies using conditional alleles demonstrate that the expression of *Calcrl* in ECs is required for survival, but that its expression in cardiomyocytes and VSMCs is dispersible.

A number of studies have described both AM and CGRP as regulators of vascular tone by functioning as potent vasodilators. Moreover, these data have supported a cardio-protective role in blood pressure regulation since plasma levels of both AM and CGRP are elevated during cardiovascular stresses including hypertension, pregnancy, and congestive heart failure (rev. in [2, 17]). However, studies utilizing gene-targeted knockout mice of the AM/CGRP signaling components have failed to support an essential role for AM or CGRP in regulating basal blood pressure. Two groups have independently found that modulating either *AM*, *Cgrpα*, or *Ramp*1 gene expression resulted in significant changes in basal blood pressure [10, 38-40]. In contrast, several other groups, including our laboratory, have failed to identify a significant role for AM or CGRP in regulating basal blood pressure. For instance, we found that a genetic reduction or a genetic increase in AM expression levels had no effect on basal vascular tone [41]. Lu JT *et al*

exhaustively characterized mice lacking *Cgrpα* and found no impairment of basal cardiovascular function [42]. Further, decreased expression of *Calcrl* and *Ramp2* and global loss *Ramp1* and *Ramp3* did not affect basal blood pressure [[43], Li M in preparation]. Transgenic over-expression studies of C*alcrl* and *Ramp2* in VSMCs revealed no change in basal blood pressure relative to control mice [44, 45]. In the present investigation, we found no differences in basal blood pressures and cardiovascular functions between VSMC-specific *Calcrl* deficient mice and controls. These data continue to support the concept that AM and CGRP signaling through VSMCs is dispensable for the regulation of basal vascular tone.

Interestingly, VSMC-specific *Calcrl* deficient mice were found to be less sensitive to catecholomine mediated vasoconstriction when compared to controls. Since hemodynamic parameters are highly regulated through a variety of neuromuscular and hormonal mechanisms, these data would suggest that other mechanisms may be in place to compensate for the lack of two major vasodilators, by downregulating vasoconstrictor activity. At first glance, these data would suggest that alpha adrenergic receptor expression may be down regulated in VSMC-specific *Calcrl* deficient mice lending to the decreased responsiveness to phenylephrine. Alternatively, since AM and CGRP generate a vasodilator response in VSMCs through a cAMP-mediated increase in activation of ATP-sensitive potassium channels and decreases in intracellular calcium levels (rev. in [17]), it would be interesting to investigate whether other interacting signaling cascades have been effected by loss of CLR.

In conclusion, this study details this first investigation to specifically examine the role of CLR signaling in VSMCs through generation of VSMC-specific *Calcrl* deficient mouse model. We have found no difference in the initial characterization of knockout mice compared to control, suggesting CLR signaling is not necessary for the development or basal cardiovascular function in mouse models.

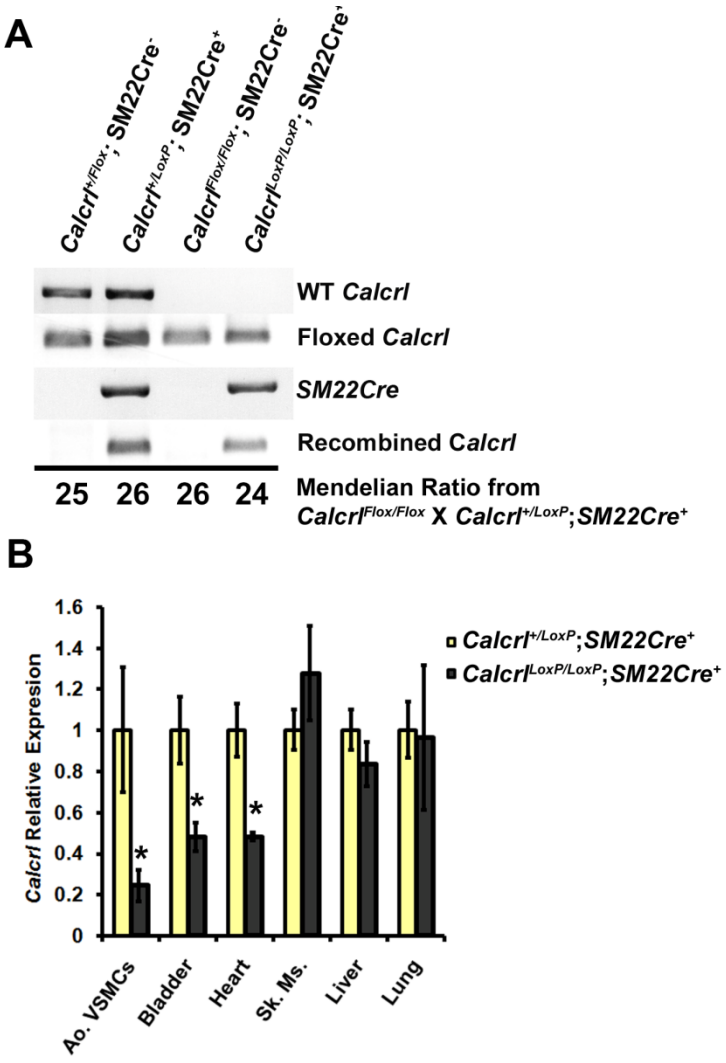

Figure 5.1: Generation of VSMC-specific *Calcrl* deficient mice. (**A**) PCR representing the four genotypes generated from the parent cross of *Calcrl*$^{Flox/Flox}$ x *Caclrl*$^{+/LoxP}$;*SM22Cre*$^{+}$. The recombined "floxed" allele is only detected in *SM22Cre*$^{+}$ mice and all mice were born at the expected Mendelian ratios. (**B**) End-point RT-PCR results of *Calcrl* gene expression in VSMCs, Heart, and Skeletal Muscle (Sk. Ms.) tissues collected from control and *Calcrl*$^{LoxP/LoxP}$;*SM22Cre*$^{+}$ animals. A significant reduction in VSMC *Calcrl* gene expression is found in *Calcrl*$^{LoxP/LoxP}$;*SM22Cre*$^{+}$.

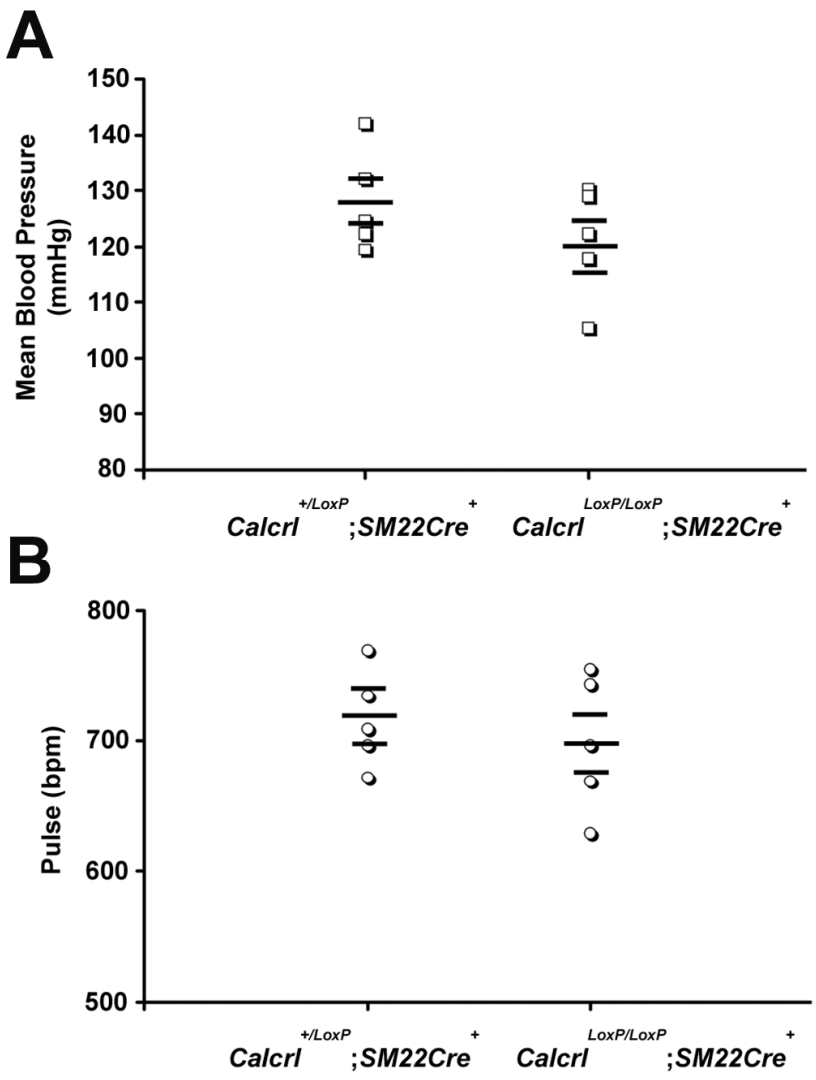

Figure 5.2: Assessment of Basal Cardiovascular Function. (**A**) Mean blood pressure of conscious *Calcrl*$^{+/LoxP}$;*SM22Cre*$^{+}$ and *Calcrl*$^{LoxP/LoxP}$;*SM22Cre*$^{+}$ mice measured by computerized tail cuff. (**B**) Heart rate of conscious *Calcrl*$^{+/LoxP}$;*SM22Cre*$^{+}$ and *Calcrl*$^{LoxP/LoxP}$;*SM22Cre*$^{+}$ mice. No significant difference was found between the two groups (n=5).

Table 5.1

Basal echocardiographic parameters of *Calcrl*$^{+/LoxP}$; *SM22*Cre^{+} and *Calcrl*$^{LoxP/LoxP}$; *SM22*Cre^{+} mice.

	Calcrl$^{+/LoxP}$; *SM22*Cre^{+}	*Calcrl*$^{LoxP/LoxP}$; *SM22*Cre^{+}	P value
N	5	6	
Heart Rate (bpm)	510±12	478±8	0.11
LVED, d (mm)	3.75±0.16	4.00±0.11	0.24
LVED, s (mm)	2.86±0.15	3.06±0.13	0.32
LVPW, d (mm)	1.07±.0.08	0.92±0.04	0.14
LVPW, s (mm)	1.28±0.08	1.13±0.07	0.19
%FS	25.8±3.1	23.6±1.7	0.56
%EF	48.8±6.2	47.4±2.9	0.85
CO (ml/min)	16.0±2.0	15.3±0.7	0.86
AortaD, d (mm)	1.49±0.05	1.38±0.07	0.25
AortaD, s (mm)	1.29±0.07	1.22±0.06	0.69

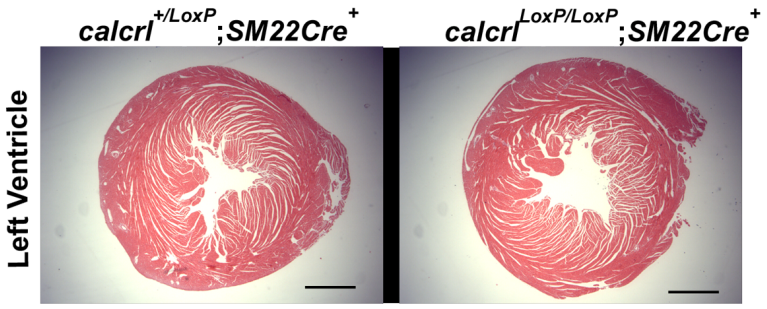

Figure 5.3: Examination of *Calcrl*$^{+/LoxP}$;*SM22Cre*$^{+}$ and *Calcrl*$^{LoxP/LoxP}$;*SM22Cre*$^{+}$ mice mouse hearts. H & E stained left ventricles reveal no histological difference the two genotypes. Bar equals 1 mm.

Table 5.2

Phenotypic Profiles of *Calcrl*$^{+/LoxP}$; *SM22*Cre^{+} and *Calcrl*$^{LoxP/LoxP}$; *SM22*Cre^{+} mice.

	Calcrl$^{+/LoxP}$; *SM22*Cre^{+}	*Calcrl*$^{LoxP/LoxP}$; *SM22*Cre^{+}	P value
Body Weight (g)	33.33±1.58	32.62±0.67	0.70
Heart vs BW ratio	5.48±0.34	5.31±0.21	0.80
Left Ventricle vs BW ratio	4.04±0.27	3.78±0.16	0.44
Water intake(24h, ml)	4.2±0.4	4.4±0.2	0.32
Urine Volume (24 h, ml	1.5± 0.2	1.4±0.3	0.88

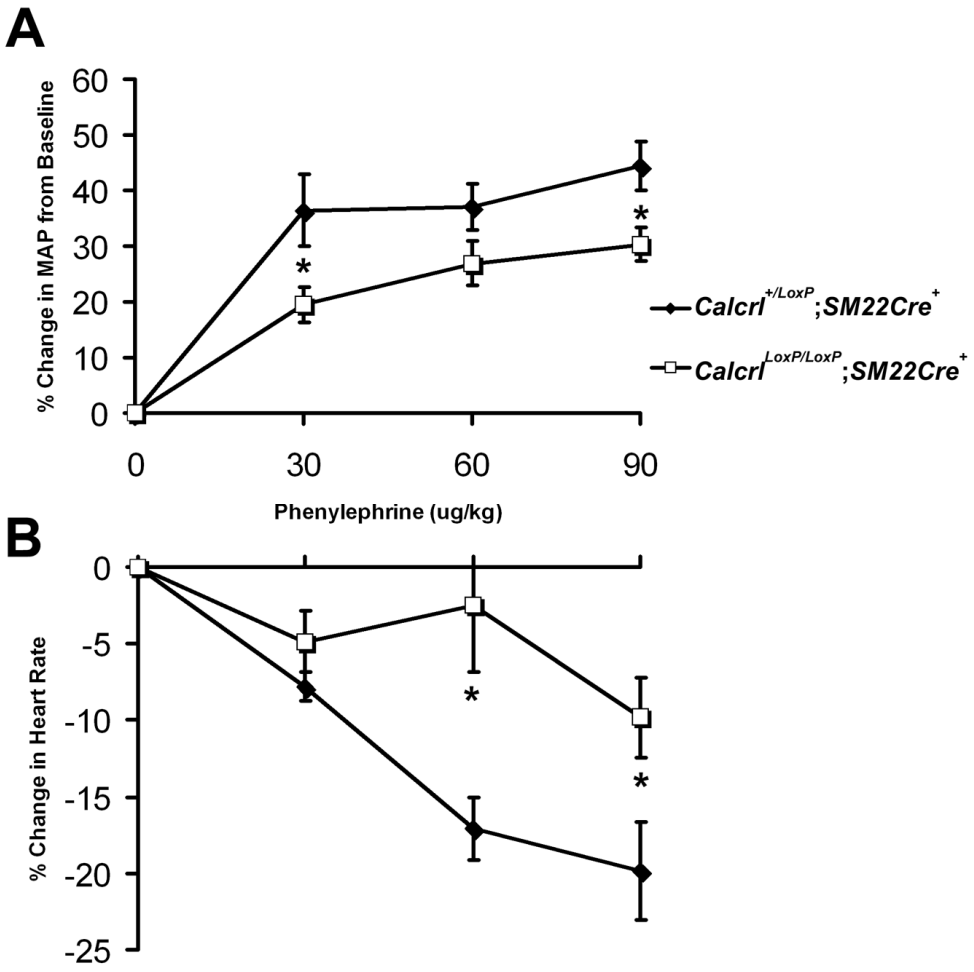

Figure 5.4: Reduced cardiovascular response in phenylephrine treated VSMC-specific *Calcrl* deficient mice. Increasing doses of phenylephrine were infused into the right jugular vein of anesthetized mice. The *Calcrl*$^{LoxP/LoxP}$;*SM22Cre*$^{+}$ mice demonstrated a decreased response to phenylephrine compared to controls in both (A) percent change in mean arterial pressure (MAP) and (B) percent change in heart rate. (n=7, $p<0.05$)

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CHAPTER 6

CONCLUSIONS & FUTURE DIRECTIONS

This thesis describes the role of adrenomedullin signaling in the development and function of the cardiovascular system with specific attention to the lymphatic vasculature and vascular smooth muscle cells. In completing this thesis, several mouse models and cell culture systems were utilized to investigate a variety of biological and physiological questions. Together, these findings represent important contributions to the field of adrenomedullin biology and will serve as building blocks for future studies. This discussion highlights the key points found in each chapter and explores future endeavors which may proven beneficial in expanding our knowledge of adrenomedullin biology.

Chapter 3

Key Points

1. AM signaling is necessary for murine lymphatic vascular development.
2. *AM*, *Ramp2*, *Calcrl* and *Calcrl* endothelial-specific null mice develop generalized edema with hypoplastic lymph sacs due to a specific and preferential reduction in lymphatic endothelial cell proliferation during development.
3. *Calcrl* is up-regulated in LECs by Prox1, suggesting that enriched AM signaling in LECs is necessary for embryonic survival.

Future Directions

The early embryonic lethality in AM signaling null mice, while informative in contributing to our understanding of the necessity of AM signaling during

development, precludes our ability to pursue functional and mechanistic questions in later development and adulthood. As such, I believe that the generation of two additional mouse models would greatly expand our understanding of AM signaling in endothelial cells. First, by utilizing the Cre-Lox system of conditional gene deletion, one could cross floxed *Caclrl* mice to a tamoxifen inducible Cre driven by an endothelial specific promoter (currently available include VE-Cadherin) [1]. Resultant mice generated from this cross would express normal *Calcrl* as a wildtype mouse, except when treated with tamoxifen, which would delete *Calcrl* specifically in endothelial cells. Thus, one could study the loss of AM signaling in endothelial cells during different developmental stages, and even in adulthood, to assess whether AM signaling in ECs is required for survival once the lymphatic vascular system has been established. Second, to examine whether AM signaling solely in ECs is sufficient for survival, one could over-express CLR in endothelial cells using an endothelial specific promoter (i.e. VE-Cadherin) and breed these mice onto the background of a global *Calcrl* null mouse by crossing to the *Calcrl* heterozygotes. A caveat to both of these proposed models is that we will be unable to distinguish between the effects of AM on blood versus lymphatic endothelium because the VE-Cadherin promoter is expressed in both lineages. At this time, a lymphatic endothelial-specific promoter has not been characterized to drive gene expression specifically in lymphatics. However, if and when this tool becomes available, it would be very interesting to generate the two aforementioned mouse models in the context of lymphatic endothelial cells. This would allow one to evaluate whether AM

signaling specifically in LECs in necessary for survival and, through subtractive analysis, permit one to distinguish the role of AM signaling in LECs versus BECs.

Deciphering the endothelial specific intracellular pathways that are modulated by AM signaling could also provide valuable insight into understanding how AM signaling elicits its cellular effects. In Chapter 3, it was shown that AM stimulation activates MAPK in LECs to set forth the transcriptional events leading to increased proliferation. It would very interesting to know which other intracellular pathways are affected by loss of AM in terms of both expression and activity. To this end, primary WT and AM null ECs could be isolated and genome-wide microarrays could be performed to identify transcriptional targets that are downstream of AM signaling. These novel molecular players could then be functionally assessed using a variety of lymphangiogenic assays including: cultured mouse thoracic ducts and corneal neovascularization assays. A highly likely potential pathway that could interact with AM signaling in LECs is the VEGF signaling cascade, which in itself is perhaps the most potent angiogenic pathway in endothelial cells. Several studies, including work from the Caron lab, have suggested that AM signaling can augment VEGF signaling, resulting in an increased angiogenic response [2, 3]. In one study, a mechanism was suggested in which AM signaling through CLR transactivates the VEGF receptor [4]. Cultured *AM* and *Calcrl* null EC, generated by stable siRNA expression, could be a valuable tool to further explore this mechanism.

Lastly, AM expression has been found to up-regulated in many forms of cancer and most likely promoted by the hypoxic conditions of the cancerous growth [5]. Currently, all of these studies have focused on the angiogenic properties of AM,

but the lymphangiogenic properties remain to be investigated. By performing immunohistochemistry on the aforementioned tumor biopsies with lymphatic endothelial specific markers, one could determine whether increased AM expression correlates with increased lymphatic vessel density. Based on the findings from this dissertation, one would expect a positive correlation of AM expression with lymphatic vessel density. Assuming this to be true, the next logical step would be to generate anti-AM therapies to fight cancer growth and metastasis. To this end, a current focus of our laboratory is in the generation and characterization of a small molecule inhibitor against AM activity which specifically targets the RAMP2-CLR extracellular domains.

Chapter 4

Key Points

1. AM stabilizes the lymphatic endothelial cell barrier in vitro by preventing VEGF mediated disruption of junctional proteins.

2. AM decreases lymphatic capillary permeability in vivo and completely abrogates VEGF increases in permeability.

Future Directions

The intracellular signaling mechanism activated by AM to stabilize the LEC barrier remains unknown. However, one model has been put forth by Hippenstiel *et al* from studies utilizing cultured blood endothelial. They found that AM stabilized the EC barrier through a cAMP dependent relation of the micro filament system,

specifically inhibiting myosin-light chain phosphorylation and stress fiber formation [6]. While a similar mechanism may exist in LECs, we were unable to replicate these findings in the context of AM attenuating VEGF-mediated increases in permeability (data not shown). Regardless, while the intracellular signaling mechanism may be unclear, the dramatic ability of AM to stabilize junctional proteins like VE-cadherin and ZO-1 at the plasma membrane in both LECs and BECs should be further investigated. Indeed, the state of tyrosine phosphorylation of cadherins and their associated proteins can greatly influence the stability of adhaerens junctions [7, 8]. For instance, it has been well established that VEGF signaling through VEGFR-2 results in a phosphorylation event that directly leads to endocytosis of VE-cadherin and decreased barrier stability (i.e. increased permeability) [9]. Further, it has been suggested that the activities of RAP1, a RAS family GTPase, may play a vital role in cAMP directed stabilization of junctional complexes at the plasma membrane, specifically VE-cadherin [10]. As such, it may be worthwhile to explore the effect of AM signaling on junctional protein phosphorylation or RAP1 activation, in the hopes of providing a mechanism for our observations.

While the data presented in Chapter 3 demonstrate that AM functions to stabilize the lymphatic endothelial cell barrier in vitro and in vivo, it remains to be shown whether this function holds true during pathological conditions such as lymphedema, tumor metastasis, or dendritic cell trafficking during the immune response. Generation of mouse models which specifically expresses *Calcrl* in the lymphatic endothelium at varying doses would greatly facilitate such studies. Our

lab has recently generated a "gene-titration" mouse for the *AM* gene which could be applied to the *Calcrl* as well. Briefly, the 3' UTR of the endogenous AM gene was replaced with a 3' UTR of bovine growth hormone (*bGH*) encompassed by flox sites and followed by a stop codon, which highly stabilized the *AM* mRNA transcipt leading to an increase mRNA half-life and a nearly three-fold increase in *AM* expression. Further 3' to the *bGH* region, the 3' UTR of the *C-fos* gene, a highly unstable mRNA with a short half-life, was engineered into the targeting cassette as well. Introduction of Cre recombinase excises the stabilizing *bGH* region, allowing transciption of the unstable *C-fos* region, resulting to a highly unstable *AM* mRNA transcript. It would be very interesting to utilize the same approach for *Calcrl* and introduce Cre recombinase driven by a lymphatic endothelial-specific promoter, therefore generating mice with modulated AM signaling by either over-expressing *Calcrl* or under-expressing *Calcrl* in the lymphatic endothelium. These mice could then be utilized for studies investigating the role of AM signaling in the lymphatic endothelium during pathological conditions.

Chapter 5

Key Points

1. Since global knockouts of AM signaling components are embryonic lethal, VSMC-specific *Calcrl* knockouts were generated to determine the cell-specific cause of embryonic lethality. This was accomplished by introducing Cre recombinase driven by the VMSC-specific promoter SM22 into the background of “floxed” *Calcrl* mice.

2. VSMC-specific *Calcrl* deficient mice were born at expected Mendelian ratios and survived to adulthood with no obvious functional defects. Therefore, the CLR mediated signaling events of AM or CGRP ligand binding are dispensable for murine vascular development.
3. Consistent with our previous studies, we observed no differences in basal blood pressure or cardiac function in VSMC-specific *Calcrl* deficient mice compared to control mice. Thus, CLR mediated signaling events are not essential for maintaining basal hemodynamics.
4. Unexpectedly, VSMC-specific *Calcrl* deficient mice displayed a reduced response to induced vasoconstriction by the α-adrenergic receptor agonist phenylephrine compared to control mice. This suggest that other hemodynamic regulators may have compensated for the loss of AM and CGRP signaling in the vascular smooth muscle.

Future Directions

Over the past decade much debate has been generated on whether AM and CGRP function as essential regulators of basal vascular tone. To date, at least 8 independent studies have specifically examined either AM or CGRP signaling in genetic altered mice including *AM*, *Calcrl*, and *Ramp2* heterozygote mice, *CGRP* knockout mice, VSMC-specific *Calcrl* over-expressing mice, VSMC-specific *Ramp2* over-expressing mice, and global knockouts of *Ramp1* and *Ramp3* [10-19] . The majority of these studies, with the exception studies from two laboratories, have rebuked the hypothesis that AM and CGRP are potent vasodilators contributing to

basal hemodynamics. The present study directly addressed this debate through the generation and characterization of VSMC-specific *Calcrl* deficient mice. Our results strongly confirm a non-essential role for AM and CGRP signaling in the regulation of basal blood pressure.

However, the role of these signaling pathways during pathological conditions remains to be resolved. Therefore, we chose to challenge the VSMC-specific *Calcrl* deficient mice with pharmacological vasoconstriction, expecting the mice would show greater vasoconstriction in the absence of two potent endogenous vasodilators. Unexpectedly, we found a decreased change in mean arterial pressure after stimulation with phenylephrine in the VSMC-specific *Calcrl* deficient mice. These results suggest that other hemodynamic mechanisms may be compensating for the loss of AM and CGRP vasodilator functions in the VSMC-specific *Calcrl* mice.

It would be interesting to extend these studies to mouse models of acute hypertension by aortic constriction or chronic hypertension through the introduction of the renin transgene [20] to elucidate the role of AM and CGRP signaling in VSMCs during the diseased state. In addition, these proposed studies would allow us to challenge the postulated compensatory mechanisms over an extended period of time.

The lack of an observed hypertensive response in the VSMC-specific *Calcrl* deficient mice also leaves open the possibility that AM and CGRP signaling in the endothelium could be solely responsible for coordinating the vasodilator activities of AM and CGRP most likely through a nitric oxide-dependent mechanism (see **Figure**

1.3). It is currently difficult to address this hypothesis directly because the endothelial-specific *Calcrl* mice die during embryogenesis. However, utilization of an endothelial specific tamoxifen-inducible Cre mouse [1], to excise *Calcrl* postnatally from the vascular endothelium could serve as a very powerful tool.

Because the functional role of CLR in mediating smooth muscle cell tone may be less relevant to the vasculature but more important in other tissues like the lung and the uterus, the current VSMC-specific *Calcrl* deficient mouse may be useful to study pathological conditions in these tissues.

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APPENDIX

SUPPLEMENTARY INFORMATION FROM CHAPTER 3: ADRENOMEDULLIN SIGNALING IS ESSENTIAL FOR MURINE LYMPHATIC VASCULAR DEVELOPMENT

Supplemental Expanded Methods

Tissue Preparation, Immunohistochemistry and Microscopy

Tissues or cells, plated on glass coverslips, were fixed in 4% paraformaldehyde in PBS overnight. For immunofluorescent staining, tissues were then cryoprotected with 30% sucrose in PBS overnight, embedded in OCT (Tissue-Tek) and cryosectioned at 6-8µm. Sections or cells were rehydrated in PBS, permeablized with 0.4% Triton-X100 in PBS, blocked with 4% BSA in PBS and incubated in primary antibody overnight. After washing, fluorescently labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were incubated for 2 hours at room temperature, sections were washed and mounted for imaging. EGFP was imaged immediately after permeablization. Images were acquired on a Nikon E800 microscope with a Hammamatsu ORCA-ER CCD camera using Metamorph Software (Molecluar Devices) and processed in Photoshop8.0 (Adobe). For hematoxylin and eosin staining, fixed tissues were put through an ethanol gradient, embedded in paraffin and sectioned at 10µm. Sections were deparaffinized and stained with Mayer's hematoxylin and eosin following standard protocols.

Quantitative RT-PCR

Lymphatic vessels were isolated from the hind limbs of adult SvEv129/6 mice. Briefly, the hind footpads of anesthetized mice were injected with 3% Chicago sky blue in Tyrode's Buffer. After incorporation, mice were euthanized and lymphatic vessels labeled with blue dye were excised under a dissecting microscope. RNA

was isolated using the RNeasy Micro kit (Qiagen, Valencia, CA) with additional DNAse treatment (Promega). RNA from embryos and cultured cells was isolated using the RNeasy Mini kit. cDNA was then generated using the iScript Select cDNA synthesis kit (Bio-Rad, Hurcules, CA).

Quantitative PCR was performed on the Stratagene Mx-3000p machine (La Jolla, CA) using ABgene ROX master mix (Rochester, NY) and rodent GAPDH control reagents (Applied Biosystems, Foster City, CA). Relative levels of gene expression were determined using the comparative quantitation ($\Delta\Delta$CT) method with MxPro software (Stratagene). Sequences for the mouse *AM*, *calcrl* and *RAMP2* primers and probes have been published (33-35) and all other primer and probe sequences are provided in Supplemental Figure 6 online. All assays were repeated at least twice, and all samples were run in triplicate. Results were analyzed using the two-tailed Student's t-test assuming unequal variance.

Interstitial fluid analysis

To collect interstitial fluid from homozygous null embryos, *RAMP2*$^{-/-}$ E14.5 embryos were dissected in PBS. A 30.5 gauge needle was inserted under the edematous skin and fluid was slowly drained. Rat lymph was collected directly from the thoracic duct of an adult Sprague-Dawley rat and mouse serum was collected by tail bleed from adult SvEv129/6 mice. Samples were centrifuged at 30,000x g and supernatants were loaded onto 8-12% gradient polyacrlymide tris-glycine gels. Gels were stained with coomassie blue 250 (Sigma.)

Quantification of Lymph Sac Area

Hematoxylin and eosin stained transverse sections of the jugular region of *AM, calcrl and RAMP2* null embryos were imaged on a Leica MZ16 dissecting microscope. Sections containing jugular lymph sacs that were matched for the same anteroposterior level were blindly measured using ImageJ software (NIH, USA). In order to normalize for section variability, the area of the lymph sac (defined as the area within the perimeter) was divided by the area of the adjacent jugular vein.

Transmission Electron Microscopy

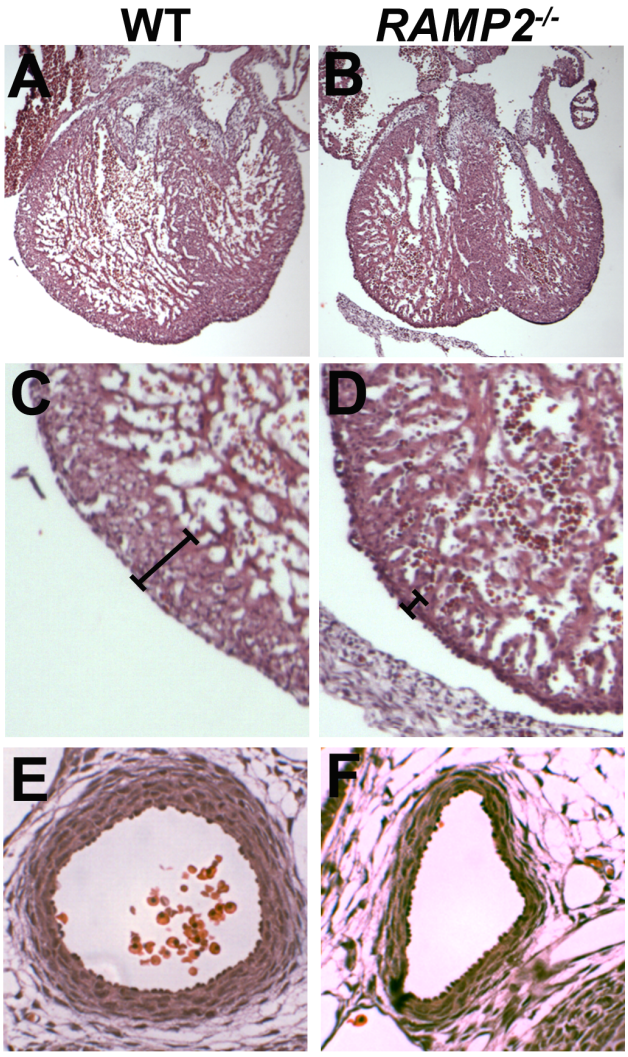
Embryos were collected and fixed in 2% formaldehyde/2.5% glutaraldehyde in 0.15M sodium phosphate buffer, pH 7.4, overnight at 4˚C. Following several washes in sodium phosphate buffer, the samples were post-fixed for 1 hour in potassium ferrocyanide-reduced osmium, dehydrated through a graded series of ethanol and embedded in PolyBed 812 epoxy resin (Polysciences, Warrington, PA). Using a diamond knife, 1 □m cross-sections were cut, stained with 1% toluidine blue and examined by light microscopy to isolate areas containing jugular lymph sacs. Ultrathin sections were cut with a diamond knife (70-80 nm thickness), mounted on 200 mesh copper grids and stained with 4% aqueous uranyl acetate for 15 minutes followed by Reynolds' lead citrate for 8 minutes. The sections were observed using a LEO EM-910 transmission electron microscope (LEO Electron Microscopy, Inc., Thornwood, NY), accelerating voltage of 80 kV, and micrographs were taken using a Gatan Orius SC 1000 CCD Camera (Gatan, Inc., Pleasanton, CA).

In Cell Western

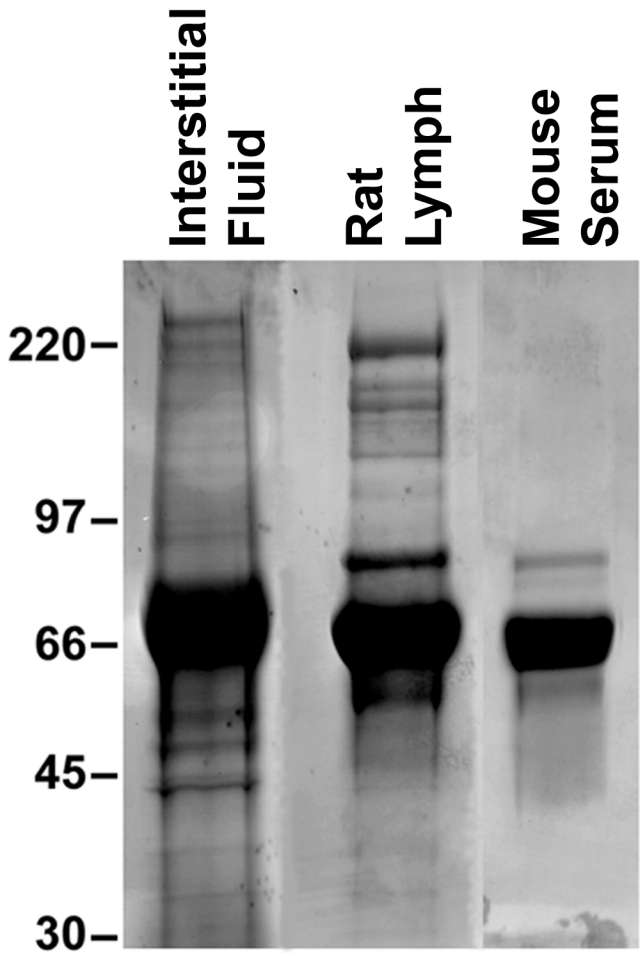
$8x10^4$ cells/ml were cultured under normal growth conditions and after 24 hours media was changed to basic medium containing 0.5% FBS for 24 hours. Cells were then treated with 10nm AM for indicated times. At the end of incubation, the medium was removed and the cells were fixed, permeabilized, blocked, and treated with the appropriate antibodies per manufacturer's instructions (LI-COR). The primary antibodies, anti-phosph-ERK1/2 and anti-ERK1/2, were used at dilutions 1:100 and 1:25, respectively, and detected with goat-anti-mouse IR680 and goat-anti-rabbit IR800 each at 1:5000 dilutions. The plates were scanned with the Odyssey infrared scanner, data was obtained using scanner software and total area under the curve was determined by Gaussian best-fit analysis.

Western Blotting

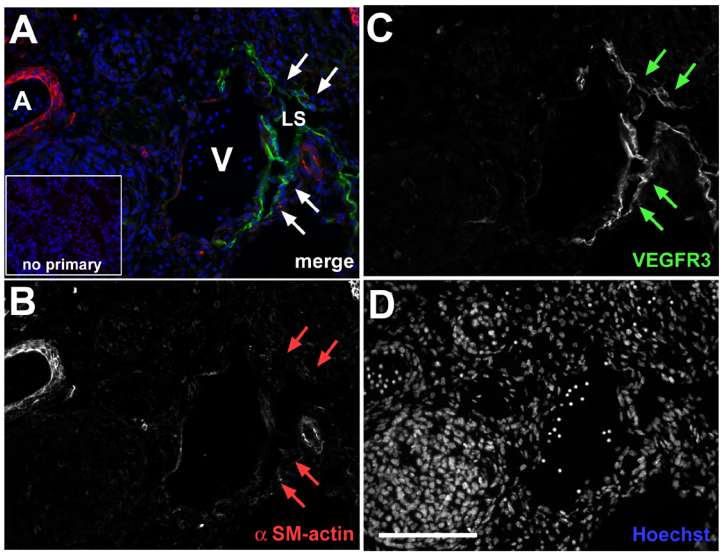
LECs were treated with 10nm AM for indicated time points, washed three times with PBS, lysed in 100µl of sample buffer, boiled for 10 min, loaded onto a 10% polyacrylimide gel and resolved by SDS-PAGE. Protein was then transferred to a polyvinylidene diflouride (PVDF) membrane which was subsequently blocked in 5% non-fat dry milk overnight at 4 degrees. The primary antibodies were incubated overnight at 4 degrees at 1 mg/ml and detected with secondary antibodies as described for the In Cell Western above. The blot was imaged with the Odyssey infrared scanner and data was obtained using scanner software.



Supplemental Figure 1: Developmental cardiovascular defects of *RAMP2*$^{-/-}$ embryos phenocopies those of *AM*$^{-/-}$ and *calcrl*$^{-/-}$ embryos. Histological sections from E14.5 wildtype and *RAMP2*$^{-/-}$ embryos stained with H&E. The *RAMP2*$^{-/-}$ embryos had generally smaller hearts than wildtype littermates (A,B) with overtly normal artia, ventricles, valves and septum. The thickness of the compact zone of the right ventricle (RV) was significantly diminished in *RAMP2*$^{-/-}$ embryos compared to wildtype littermate controls (C,D). While the endothelial lining of aortas (Ao) from *RAMP2*$^{-/-}$ embryos appeared indistinguishable from that of wildtype littermates, there was a remarkable reduction in the extent of vascular smooth muscle coverage in *RAMP2*$^{-/-}$ embryos compared to wildtype (E,F). In summary, RAMP2$^{-/-}$ embryos displayed developmental cardiovascular defects similar to those previously reported for *AM*$^{-/-}$ and *calcrl*$^{-/-}$ mice.



Supplemental Figure 2: Interstitial fluid removed from *RAMP2*$^{-/-}$ embryos contains high molecular weight proteins. Fluid was removed from an E13.5 *RAMP2*$^{-/-}$ embryo and centrifuged to remove cellular debris. The supernatant was resolved on an SDS-PAGE gel along with lymph obtained directly from the thoracic duct of a rat, and mouse serum. Commassie staining shows that the edematous fluid contains an abundance of albumin (67kD) and numerous other high molecular weight proteins (>100kD), typical of lymph and unlike serum.



Supplemental Figure 3: Lymph sacs in developing E14.5 embryos are not lined by smooth muscle cells. (A-D) Immunofluorescent staining of transverse sections through the jugular region of WT embryos. Newly formed lymph sacs (LS) which are lined with VEGFR3-containing endothelial cells (panel A:green, panel C) are not surrounded by smooth muscle, as indicated by lack of a SM-actin staining (panel A:red, panel B:arrows). Smooth muscle staining can be seen around the carotid artery (A) and jugular vein (V) of panels A and B. Hoechst stained nuclei are shown in panel D and indicated in blue in panel A. Inset in panel A shows no primary antibody control.

Supplemental Movie 4: High resolution, signal volume rendered, 3-Dimensional movie of fluorescent immunohistochemistry on an E14.5 wildtype embryo using VEGFR3 antibody to visualize lymphatic vascular development. This movie can be viewed at http://www.jci.org/articles/view/33302/sd/2.

Supplemental Movie 5: High resolution, signal volume rendered, 3-Dimensional movie of fluorescent immunohistochemistry on an E14.5 *RAMP2*$^{-/-}$ embryo using VEGFR3 antibody to visualize lymphatic vascular development. This movie can be viewed at http://www.jci.org/articles/view/33302/sd/3.

ES/Mouse Genotyping

	Primer Name	Primer Sequence (5' to 3')	Product size
AM	AM Forward	gtgctgacgggatcgtgctg	WT: 600
	AM Reverse	catgcagtacccgagggacct	KO: 400
	GFP Reverse	gggtcttgtagttgccgtcgt	
RAMP2	R2 Forward	tctgtctggatgctgccttgc	WT: 900
	R2 Reverse	gaagtcaggcagtcagggttg	KO: 675
	Neo Reverse	ttctatcgccttcttgacgagttc	
Calcrl	CLR Forward	gcggagcatattcaatcacaag	WT: 967
	CLR Reverse	gaaatgtgctgtatgttcaagc	KO: 800
	Neo Reverse	tggcggaccgctatcaggac	
Tie2-Cre	Cre Forward	ggcatggtgcaagttgaata	Cre: 1400
	Cre Reverse	ctggggtcttctacctttctctt	
Calcrl Flox	P1	cagattagctcagctgtatcacac	WT: 540
	P2	gcggagcatattcaatcacaag	Flox: 230
	P3	gaaatgtgctgtatgttcaagc	
	P4	ttctatcgccttcttgacgagttc	

Human Q-RT-PCR TaqMan

	Primer Name	Primer Sequence (5' to 3')	Product size
AM	hAM Forward	agctcaagccttgccacttc	163
	hAM Reverse	gacggaaaccagattcatcc	
	hAM Probe	ttagcagggtctgcgcttcgca	
RAMP2	hR2 Forward	gaaaaggcttggtgcgactg	153
	hR2 Reverse	gttggcaaagtggatctggt	
	hR2 Probe	agcaggccttatagcaccctgcga	
CALCRL	hCLR Forward	gcgacctgaaggaaagattg	123
	hCLR Reverse	agaattgcttgaacctctcca	
	hCLR Probe	tgcacttccagggtctttggtctcac	
LYVE1	hLyve1 Forwarc	gcaccatgtctacagaaactgaa	
	hLyve1 Reverse	gcagcaccaaagaagaggag	
	HLyve1 Probe	atgaagctgctgggtttggaggtgt	

Supplemental Figure 6: Primers and Probes used for Genotyping and Gene Expression Analysis.